April 2019

Assessing the Nutritional Value of Black Soldier Fly Larvae (Hermetia illucens) Used for Reptile Foods

Kimberly L. Boykin
Louisiana State University and Agricultural and Mechanical College, kimkatnc1@gmail.com

ASSESSING THE NUTRITIONAL VALUE OF BLACK SOLDIER FLY LARVAE (*HERMETIA ILLUCENS*) USED FOR REPTILE FOODS

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Master of Science

in

The Department of Veterinary Sciences

by
Kimberly Lynne Boykin
B.S., Florida State University, 2008
D.V.M., North Carolina State University, 2016
May 2019

ACKNOWLEDGEMENTS

I am greatly indebted to a number of individuals whose expertise and support have been instrumental in helping me reach the completion of my thesis research. Foremost, I would like to express my sincerest gratitude to my major professor/advisor/ life coach, Dr. Mark Mitchell. If it were not for his continued enthusiasm, motivation, and guidance, I would not be where I am today. He truly took me under his wing and has taught me how to become the best clinician scientist I can be. I could not have imagined having a better advisor and mentor for my graduate studies.

I would also like to thank the rest of my thesis committee, Drs. Thomas Tully and Rhett Stout for their encouragement, insightful comments, and guidance through the completion of this project.

My sincerest thanks also go to all of the wonderful people at Fluker's Cricket Farm. David, Waco, Vance, Casey, and Sam have been the best cheerleaders a girl could ask for. Their support of these projects was instrumental and I would never have been able to complete them without their help. The reptiles in the projects would also like to express their thanks for all of the yummy insects you guys provided!

I thank my fellow labmates and team members within the Zoological Medicine service including Drs. Laure Molitor, Sean Perry, Anke Stöhr, Kelly Rockwell, Kyra Berg, Javier Nevarez, and Ms. Jordan Padlo, for their assistance in projects and for providing me with contacts, knowledge, or skills to further develop as a veterinarian. Their friendship and support has been indispensable during these last two years.

I must express a special gratitude to the veterinary students and staff within the Department of Laboratory Animal Medicine who spent countless hours helping me to care for the numerous animals that were a part of this project. To Karina Butler-Perez, Jordan Peters, and Cameron Buck, thank you from the bottom of my heart for all of your hard work and for allowing me a few days off every now and then. I greatly enjoyed the time we got to spend with each other and wish you all the best in your future careers. To Janelle Allen, thank you for your assistance and patience while having to deal with so many reptiles and escaped insects!

To everyone at Louisiana State University's School of Veterinary Medicine that I have had the privilege to work with during my internship and graduate program, thank you for your friendship, mentorship, and scholarship. My time at LSU has been a wonderful experience and I appreciate all of the things that I have gotten to do and learn during my time here. Special thanks are in order to Dr. Renee Carter for her assistance with one of the projects and to Drs. Rebekah Gallaher and Amy Grooters for their continued interests in my research pursuits.

Finally, my deepest gratitude is for my family. They have supported and loved me in all that I have done in my life, even if they have not fully understood the full extent of my research projects nor the reasons for doing them. Most importantly, thank you to my fiancé Chad, who has patiently endured countless nights and weekends interrupted by research pursuits and the endless amount of writing that has accompanied it. Thank you for believing in me and supporting me through this journey. I love you all.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Black soldier fly (BSF) larvae have been studied extensively in animal feed production, but there is limited research for non-production species, such as reptiles, despite their popularity as a calcium-rich feeder insect. The goals of this thesis were to determine the nutritive value of BSF larvae for a lizard species and as an ingredient in a diet formulated for snakes.

BSF larvae are deficient in fat soluble vitamins (A, D, and E). Using vitamin A as a test nutrient, several factors were identified that affect the success and consistency of gut loading. Based on the results, the following gut loading recommendations were established: vitamin A concentration of the larval diet should be between 16,000-20,000 mcg/kg, gut loading time period should be 24 hours, dietary moisture content should be between 56-65%, and larval density should be between 0.1-1 larvae/g of moist substrate.

Previous research in amphibians has suggested that BSF larvae have low digestibility. Using leopard geckos as a model, gut loaded and non-gut loaded larvae were fed as the primary diet for six months. Compared to the amphibian study, all nutrients were significantly more digestible, with the exception of calcium, as it likely remained bound to the undigested portions of the exoskeleton. Biochemistry results also revealed a possible calcium deficit occurring over time. Gut loaded vitamin A was confirmed to be digested as plasma and liver vitamin A concentrations were significantly higher in the gut loaded group (plasma: $t=1.906$, $p=0.0415$; liver: $t=1.951$, $p=0.0325$).

For carnivorous reptiles, an experimental sausage diet with BSF larvae as the primary protein was fed to juvenile corn snakes. The diet was fed for two months and compared to a diet of frozen-thawed mice. There was no significant difference between

diet groups regarding health or growth parameters, suggesting that BSF larvae could be used to feed snakes.

The results of this work support the idea that BSF larvae are not a good source of calcium in their natural state, but that they can be gut loaded or used in a complete feed diet to provide the nutrients needed by reptiles to support health and growth.

CHAPTER 1.
INTRODUCTION

Black soldier fly (BSF) larvae were first marketed for reptiles in 2005 as Phoenix Worms® (Insect Science Resource LLC, Tifton, GA). The founder of the Phoenix Worm brand is an avid herpetoculturist and reptile food producer, who wanted to bring a calcium-rich feeder insect to the reptile market ("Phoenix Worms®," 2019). Nutritional diseases related to low calcium diets and inversed calcium to phosphorus ratios are a common problem for captive reptiles, and are the direct result of the poor nutritional qualities of the current feeder insect market. Crickets and mealworms, which make up a majority of the market, have severely inversed calcium to phosphorus ratios of 1:7 and 1:17, respectively (Finke, 2002). Physiologically, reptiles require a much more balanced and positive ratio, with diets in the 1:1 to 3:1 range (Boyer and Scott, 2019a).

Calcium has many important roles within the body. It is important for cell signaling pathways, release of neurotransmitters, muscle contractions, cardiac health, bone strength, enzyme activity, and blood clotting (Raiti, 2019). When calcium intake is too low or phosphorus intake is too high, the body tries to maintain homeostasis within the blood and either diverts or removes the calcium that should be stored in bones. As a result, the bones become soft or brittle. Pathological fractures occur commonly in the long bones, while the jaw bones lose their strength and become fibrous (Boyer and Scott, 2019b; Raiti, 2019). Malformations occur in the vertebral column, leading to spinal kyphoscoliosis, lordosis, or rhoecosis (Boyer and Scott; 2019b). As a result, the animals lose their ability to locomote and become dehydrated and malnourished. Gravid females can suffer from eclampsia and dystocia related to poor muscle contractions (Stahl and DeNardo, 2019). As the disease progresses, animals may experience tremors, seizures,

paresis/paralysis, and eventually die. For animals that do recover, bony malformations are likely to exist throughout their life (Boyer and Scott; 2019b).

Dusting insects with calcium carbonate powders and gut loading them on calcium-rich foods prior to feeding them to reptiles has led to many improvements in reptile health over the years, but these methods are not perfect solutions. Dusting insects can be unreliable if the insects are not consumed immediately. After only 2.5 minutes, 50% of the dust applied to crickets was found to have already fallen off or had been groomed away (Li et al., 2009). The dust also makes the insects less palatable, so reptiles may delay in eating them until a majority of the dust has fallen off. Gut loading can also lead to highly variable results. In order to reach proper quantities in the gut, insects must feed on a diet that has extremely high concentrations of calcium. This leads to decreased palatability for the insect and possible food refusal. Insects that do consume the food may experience higher rates of mortality due to nutritional toxicosis. Other factors that can introduce variability include the length of time given to gut load and the developmental cycle of the insect. Because of these limitations, finding an insect species that was more calcium-rich was necessary (Finke, 2005; Livingston et al., 2014).

With a calcium to phosphorus ratio of 2.6:1, the BSF larvae is one of only a few insect species to have been identified with a positive ratio (Finke, 2013; Dierenfeld and King, 2008). Coupled with the fact that the larvae were already being reared for food animal production, the jump to making them a popular feeder insect for reptiles was inevitable. In addition to Phoenix Worms, other companies started selling them as well; and for marketing purposes have been sold to thousands of reptile owners under the guise that they do not have to be dusted or gut loaded ("Phoenix Worms®," 2019).

Unfortunately, this statement is not true. Nutritional analyses of the larvae have revealed that they are severely deficient in fat soluble vitamins (A, D_3, and E) (Finke, 2013). If BSF larvae are to be used as a staple food, they will need to be supplemented with either gut loading or multivitamin dusting on a weekly basis.

Additionally, past research in an amphibian showed that the larvae are not well-digested and there are also many anecdotal reports of full larvae being passed undigested in the feces (Dierenfeld and King, 2008). The frog study found that only 26% of the larval dry matter was digestible and that calcium digestibility was only 44% compared to dusted crickets that were 88% digestible (Dierenfeld and King, 2008). These findings suggest that the larvae may not be a good source of calcium and that even gut loaded nutrients may not be able to help rectify the situation. Poor BSF larvae digestibility may also lead to other serious health effects such as intestinal impactions (Klaphke, 2010).

Despite the popularity of these larvae, only one study has investigated the use of these larvae in reptiles. Bodri and Cole (2008) compared the growth rates of hatchling alligators fed either a complete pelleted feed diet or dehydrated BSF larvae. The alligators grew more rapidly on the pelleted diet than on the BSF larvae. Palatability was cited as a major contributing factor, but the digestibility of the diets and the lack of fat soluble vitamins in the BSF larvae may have also been contributing factors.

Obviously, there is a paucity of evidenced-based research regarding the nutritive value of these insects in reptiles. The goal of this thesis was to further investigate their digestibility, determine whether gut loaded vitamins would be absorbed by the target species, and evaluate whether they were capable of supporting the health and growth of reptiles over time.

CHAPTER 2.
LITERATURE REVIEW

2.1. Captive Reptiles and the Current Feeder Insect Market

There are more than 10,000 species of reptiles in the world, and hundreds of these species are maintained in captivity by zoological institutions, research facilities, breeders, and hobbyists (Roll et al., 2017). The species that are kept in captivity represent a large variety of taxa, as well as diverse life strategies (Mitchell, 2009). This diversity can make it challenging to mimic the natural environments and dietary needs of these animals. Because of these limitations, diseases associated with inappropriate husbandry or nutrition are common. To ensure our success with these species, it is important that we pursue evidence-based research to determine best diet practices.

The first step to identifying the nutritional needs of a captive reptile is to understand the true nutritional requirements for that species. Unfortunately, there is a lack of evidence-based research available, and many of the nutritional recommendations made for reptiles are extrapolated from requirements for the laboratory rat (NRC, 1995). Comparing the nutritional needs of a reptile to that of a mammal is not suggested because of the physiologic differences between these groups. An ectothermic animal (e.g., reptile) may only require a tenth of the energy of an endotherm (e.g., mammal). It stands to reason that nutrient requirements would also be different based on the differences in their physiology. Additionally, the laboratory rat is an omnivorous animal, while reptiles are represented by all types of feeding strategies, including omnivory, herbivory, and carnivory. Research in other animals has shown that these feeding strategies can have a significant impact on the nutrients that are required to maintain health.

Of all of the feeding strategies employed by reptiles, insectivory is one of the hardest to manage in captivity. Captive raised insects cannot replicate the natural diets of insectivorous reptiles because of a lack of insect species variance, poor commercial insect diets, and insects being fasted prior to consumption related to either shipping travel or shelf life time (Finke, 2002; Finke, 2013; Livingston et al., 2014; Oonincx and Dierenfeld, 2012). These factors lead most commercially raised insects to be deficient in certain key nutrients that their wild counter parts are more likely to be able to provide. Nutritional analyses have been performed on most of the commonly available insects commercially-raised for food. These analyses have shown that although insects provide an excellent source of most nutrients, they are usually deficient in calcium, fat soluble vitamins (A, D_3, and E), thiamine, and omega 3-fatty acids (Finke, 2002; Finke, 2003). These deficiencies often result in the development of nutritional disorders such as nutritional secondary hyperparathyroidism (NSHP, also referred to as metabolic bone disease) and hypovitaminosis A.

To correct these deficiencies, veterinarians and herpetoculturists have employed two strategies to improve the insect's nutritional quality: dusting and gut loading. Dusting insects with calcium and multi-vitamin powders is simple and can be done immediately prior to the insects being offered as food; however, this method has been shown to be unreliable as over 50% of the dust falls off or is groomed away in under 2.5 minutes when applied to crickets (Li et al., 2009). Gut loading insects with calcium and vitamin rich diets is preferred (Allen, 1997); however, these diets can provide variable results based on the duration of gut loading, developmental cycle of the insect, nutritional aspects and quality of the gut loading diet, decreased palatability of the diet to the insect,

and higher insect mortality due to nutritional toxicosis (Finke et al., 2005; Livingston et al., 2014). Because of these limitations, finding insect prey that are more nutritionally complete is warranted.

Currently, there are only a few species of insects that can be readily purchased by reptile owners. Additional species can be purchased from specialty stores online, but their availability can be limited and often times seasonal. The species that are most commonly used as feeder insects include house crickets (*Acheta domesticus*), mealworms (*Tenebrio molitor*), superworms (*Zophobas morio*), waxworms (*Galleria mellonella*), silkworms (*Bombyx mori*), and earthworms (*Lumbricus terresstris*). Table 2.1 presents the nutritional profiles of these species in a fasted state.

Regardless of feeding strategy, most vertebrate animals require a dietary calcium intake to equal or surpass that of phosphorous intake. This table highlights how ineffective most insects are at being able to provide a proper calcium to phosphorous (Ca:P) ratio. Identifying additional feeder insects with higher amounts of available calcium and more diverse nutrient profiles is of the utmost importance in our efforts to decrease the incidence of nutritional disorders such as NSHP. Those that have been identified include black soldier fly (BSF) larvae (*Hermetia illucens*), common rough woodlice (*Porcellio scaber*), and soldier termites of the species, *Nasutitermes corniger* (Finke, 2013; Oonincx and Dierenfeld, 2012; Oyarzun et al., 1996).

In recent years, BSF larvae have gained the most popularity as a feeder insect. Due to increased research pressure from food animal producers, much more is known about the BSF life cycle and the ideal conditions for raising them. Commercial insect

Table 2.1. Proximate analysis and select minerals and vitamins found in common feeder insects on an as is basis

	Adult House Crickets (*Acheta domesticus*)	Mealworms (*Tenebrio molitor*)	Superworms (*Zophobas morio*)	Waxworms (*Galleria mellonella*)	Silkworms (*Bombyx mori*)	Earthworms (*Lumbricus terresstris*)
Moisture (%)	69.2	61.9	57.9	58.5	82.7	83.6
Crude Protein (%)	20.5	18.7	19.7	14.1	9.3	10.5
Crude Fat (%)	6.8	13.4	17.7	24.9	1.4	1.6
Calories (kcal/kg)	1402	2,056	2,423	2,747	674	708
Calcium (mg/kg)	407[a]	169[a]	177[a]	243[a]	177[a]	444[a]
Phosphorus (mg/kg)	2,950	2,850	2,370	1,950	2,370	1,590
Ca:P ratio	1 : 7.2	1 : 16.9	1 : 13.4	1 : 8	1 : 13.4	1 : 3.6
Magnesium (mg/kg)	337	801	498	316	498	136
Sodium (mg/kg)	1,340	537	475	165[a]	475	965
Potassium (mg/kg)	3,470	3,410	3,160	2,210	3,160	1,820
Iron (mg/kg)	19.3	20.6	16.5	20.9	16.5	50.4
Zinc (mg/kg)	67.1	52.0	30.7	25.4	30.7	17.7
Copper (mg/kg)	6.2	6.1	3.6	3.8	3.6	1.5
Manganese (mg/kg)	11.5	5.2	4.3	1.3[a]	4.3	1.3[a]
Vitamin A (µg/kg)	<300[a]	<300[a]	<300[a]	<300[a]	474	<300[a]
Vitamin D_3 (IU/kg)	<256[a]	<256[a]	<256[a]	<256[a]	<256[a]	<256[a]
Vitamin E (IU/kg)	19.7	<5.0[a]	7.7[a]	13.3	8.9	NA

[a]Value is below the NRC requirements for rats for growth
Chart adapted from Finke, 2002

farms are now capable of successful and economical mass production, which makes them much easier to acquire for the reptile community. Compared to other common feeder insects with negative Ca:P ratios, BSF larvae and prepupae have a positive Ca:P ratio of approximately 2.6:1 in a fasted state. They have also been found to be good sources of other important minerals such as magnesium, potassium, iron, manganese, and zinc (see Table 2.2). While these insects are mineral dense, they are, unfortunately, still deficient in fat soluble vitamins (A, D_3, and E) (Finke, 2013). Additional factors such as poor digestibility and palatability may also limit their usefulness as a feeder insect for reptiles (Bodri and Cole, 2007; Dierenfeld and King, 2008). These issues need to be studied in depth before BSF larvae can be deemed "the perfect feeder insect."

Table 2.2. Proximate analysis and select minerals and vitamins of BSF larvae on an as is basis

Nutrient	**BSF Larvae (*Hermetia illucens*)**
Moisture (%)	61.2
Crude Protein (%)	17.5
Crude Fat (%)	14.0
Metabolizable energy (kcal/kg)	1,994
Calcium (mg/kg)	9,340
Phosphorus (mg/kg)	3,560
Ca:P ratio	2.62 : 1
Magnesium (mg/kg)	1,740
Sodium (mg/kg)	887
Potassium (mg/kg)	4,530
Iron (mg/kg)	66.2
Zinc (mg/kg)	56.2
Copper (mg/kg)	4.03
Manganese (mg/kg)	61.8
Vitamin A (µg/kg)	<300[a]
Vitamin D_3 (IU/kg)	<256[a]
Vitamin E (IU/kg)	6.2[a]

[a]Value is less than the NRC requirement for rats for growth; Adapted from Finke, 2013

2.2. Black Soldier Fly Natural History

The BSF is a member of the Stratiomyidae family of the insect order Diptera (true flies). BSF are native to the American continents, with a native range extending from the central United States down into South America. However, due to worldwide transportation and trade, they can now be found across the globe in tropical and temperate climates (Sheppard et al., 2002; Barragan-Fonseca et al., 2017). Fortunately, they are not considered to be a pest species, even outside of their native range, due to the fact that they are not vectors for disease, the adults do not bite or sting, and the larvae are excellent decomposers of organic waste (Barragan-Fonseca et al., 2017).

BSF are holometabolous insects and undergo a complete metamorphosis throughout their life cycle (egg, larvae, pupae, adult). In the southeastern United States, three generations of flies are able to be produced during the year between late spring to early fall (Tomberlin et al., 2002). Under laboratory conditions, these flies can be produced year-round with a typical life cycle of 40 days (Barragan-Fonseca et al., 2017). Adult flies resemble wasps and are between 15-20 mm in length (Tomberlin et al., 2002; Barragan-Fonseca et al., 2017). The average adult life span is nine days. During this time, the adults will not eat and are purely focused on mating, with females capable of producing over 500 eggs per clutch (Barragan-Fonseca et al., 2017; Tomberlin et al., 2002). Eggs hatch approximately 4 days later, with the first of six larval instars emerging (Sheppard et al., 2002). On average, the black soldier fly has a larval development time of 24 days, with larval molts occurring every four days. However, if they are given ideal conditions, they can develop in 16 days with molts occurring every 2.5 days (Barragan-Fonseca et al., 2017; Diener et al., 2009). The first five larval instars are voracious eaters

and are able to digest more than twice their own body weight in the span of a single day (Barragan-Fonseca et al., 2017). They are generalist decomposers and will readily grow on several different types of waste material, including animal manure and carrion (Tomberlin et al., 2009). Larvae will grow to an average maximum size of 0.158 grams (Barragan-Fonseca et al., 2017). During the last larval stage, or prepupal stage, feeding slows down and their cuticle starts to turn black as they start preparing for pupation. One of the primary reasons BSF larvae are used for animal feed production is because the prepupae are considered self-harvesters (Sheppard et al., 2002). The prepupae climb out of their diet substrate and will either collect themselves into a separate container or will cling to the walls of the primary container, allowing for easy collection. The prepupae weigh slightly less than the 5^{th} instar larvae due to the decrease in feeding, but calcium stores are at their highest. The calcium is accumulated in the cuticle to add strength to the soon to be developed puparium. The insect spends approximately 14 days as a pupa before shedding the highly-calcified puparium to become an adult fly with minimal calcium stores (Barragan-Fonseca et al., 2017).

2.3. Factors Affecting the Black Soldier Fly Life Cycle

The BSF life cycle has been studied and manipulated extensively by researchers in order to determine the best rearing conditions for increased fecundity, larval size, and feed conversion, as well as to shorten generational time. Temperature, relative humidity, diet composition, substrate moisture, larval density, light exposure, and light intensity have all been shown to have significant impacts on the rearing of black soldier flies, and with exception of the last two, each will be discussed in the following paragraphs (Barragan-Fonseca et al., 2017; Cammack and Tomberlin, 2017; Diener et al., 2009;

Holmes et al., 2016; Sheppard et al., 2002; Tomberlin et al., 2002; Tomberlin et al., 2009). If BSF larvae are to be used as a feeder insect for reptiles, it is important to know which factors may play a role in the process of raising and gut loading them.

The ideal temperature for rearing BSF larvae has been published to be 26-27°C (79-81°F); however, these insects are capable of being raised in temperatures ranging from 12-36°C (54-97°F) (Barragan-Fonseca et al., 2017; Holmes et al., 2016; Sheppard et al., 2002; Tomberlin et al., 2009). The ideal temperature range was determined by Tomberlin et al. (2009) by evaluating development time, larval and adult weights, and adult longevity. These factors all play a role in the reproductive ability of the adult. Although higher temperatures (30°C) resulted in faster larval and pupal development times, it also resulted in adults that weighed less and who lived for a shorter period of time than those raised at cooler temperatures (27°C). With even hotter temperatures (36°C), larvae again developed faster, but adult emergence from the pupae stage was only 0.1%. With warmer temperatures having such a strong effect on adult survivorship and their ability to lay eggs, 27°C was determined to be the ideal temperature for BSF rearing, however, higher temperatures could be beneficial if the only goal was to raise larvae to an ideal weight quickly.

The literature lists the ideal humidity for rearing BSF to be between 60-70%, but they will tolerate a range of 25-99% (Holmes et al., 2012; Barragan-Fonseca et al., 2018). Humidity levels between 60-70% produced egg eclosion rates between 72-86% and adult emergence rates of 93%. Humidity values lower than this range, such as might be seen within a heated facility during winter months, caused egg eclosion and adult emergence rates to plummet (25% humidity: egg eclosion 8%, adult emergence 16%). However, not

much difference was seen between humidity levels for the hardier larval and pupal stages, suggesting this factor is not as important for larval development and feeding rates (Holmes et al., 2012).

Diet composition is one of the more extensively studied subjects for BSF larval rearing and production. As generalist decomposers, they are capable of being raised on several different substrates, including layer hen rations, post-consumer food wastes, and even animal feces. Based on the diet they receive, their nutritional profile and life history traits can be altered (Barragan-Fonseca et al., 2017; Cammack and Tomberlin, 2017; Sheppard et al., 2002; Tomberlin et al., 2002). The type of diet may also change the larvae's rate of feeding, effecting their feed conversion ability and gut loading speeds. This subject will be discussed in more detail in a later section.

Unlike the mealworm that is usually fed a dry grain substrate, BSF larvae prefer a moist diet substrate for feeding and oviposition (Tomberlin et al., 2002). Previous research has suggested that substrate moistures should be between 52-70% (Barragan-Fonseca et al., 2017). Larval and adult weights were the heaviest when given diets with 70% moisture, whereas oviposition was most successful in moistures between 40-60% (Fatchurochim et al., 1989). As for larval density, previous research has indicated that 10 larvae per gram of diet yields the best trade-off between waste reduction efficiency and increased larval weight (Diener et al., 2009). Altogether, studies have looked at densities between 0.3-80 larvae per gram of diet and have found that larvae fed at lower densities have increased weights and shorter development times compared to larvae at higher densities that must feed on and utilize more of their own waste products (Barragan-Fonseca et al., 2017; Sheppard et al., 2002).

2.4. Different Uses for Black Soldier Fly Larvae

Over the years, BSF larvae have been used for a number of different purposes. As part of a simple, backyard setting, they can be used as composters of organic waste or as feed for omnivorous animals, such as chickens. However, with our increased knowledge regarding their high feed conversion efficiency and nutritive value, we have found more complex uses for them.

The most widely studied usage for BSF is focused on integrated waste management (Diener et al., 2009; Lalander et al., 2013; Lalander et al., 2015; Oonincx et al., 2015). It is well documented that BSF larvae are effective decomposers of organic waste. As such, they can be used to breakdown large quantities of post-consumer waste or animal manure. For over thirty years, animal producers have been using these larvae to ingest swine, poultry, and cow manure (Hale, 1973; Newton et al., 1977). The larvae efficiently turn the waste products into an edible and high quality source of animal protein that can then be fed back to those animals or others. Without the larvae, the cost of production would be higher because of the additional expenses for animal waste disposal and feeding costs (Barragan-Fonseca et al., 2017). As the world's population continues to grow and the economics of production tighten, more industries will need to adopt integrated and circular waste management options to compete.

Another benefit associated with BSF larvae is that they can serve as a “filter” to reduce potential pathogens in a production system. Erickson et al. (2004) inoculated *E.coli* O157:H7 and *Salmonella enterica* serovar Enteritidis into cow, swine, and chicken manure managed as single batch systems. After 2 days, *E. coli* growth in the chicken manure underwent a 5-log reduction compared to the control manure, and by the third

day no bacterial growth could be detected. After 2 days in *Salmonella*-infected chicken manure, the larvae had caused a 4-log reduction in growth as compared to the control. Smaller declines in growth were seen between two and four days, but never reached levels of being undetectable. The researchers confirmed that other microbial populations were not contributing to the *Salmonella* decline by repeating the experiment with autoclaved fecal material prior to *Salmonella* inoculation. Unfortunately, no declines in bacterial growth were seen in the more acidic cow and swine manure using the single batch system. However, in 2015, Lalander et al. did show a reduction in *Salmonella* Typhimurium, as well as several species of coliform bacteria, when larvae were managed on a combination of swine manure, human feces, and dog food (representing organic food waste) within a continuous stream reactor. In this experiment, *Salmonella* was reduced from 10^7 CFU/gram to below the detection limit of 1 CFU/gram despite re-infection every week when new waste material was added to the reactor. Non-specified coliform bacterial species were also reduced over time, but never eliminated from the waste material.

The reduction of pathogenic bacteria in the waste material allows for the remaining waste residue (especially from animal manure) to be further utilized as crop fertilizer (Erickson et al., 2004; Lalander et al., 2013; Lalander et al., 2015). This allows for even further integrative management and circularity of this system. Additional composting and treatment steps may still be needed to completely reduce the risk of horizontal pathogen transmission, but the larvae can be used to help reduce bulk, limit pathogen overgrowth, and reduce production costs (Lalander et al., 2013; Lalander et al., 2015). Previous research has shown that the larvae may harbor the bacteria within their

gut once collected, but the risk of transmission to consumers is relatively low as long as the larvae are dried or processed into meal prior to being used (Lalander et al., 2013).

As an animal feed, BSF larvae can be fed live, dehydrated, or ground into a meal for ingredient use, similar to chicken or fish meal. Unlike other sources of animal protein, BSF production is incredibly energy and space efficient, produces a much smaller carbon footprint, and is considered to be much more economical. Additionally, oil that is removed during the meal rendering process can also be used in the production of biofuel (Barragan-Fonseca et al., 2017). Chitin can also be separated out during processing and used in food production, textiles, cosmetics, and pharmaceuticals (Caligiani et al., 2018). Their use as an animal feed will be discussed in more detail in a later section.

2.5. Nutrient Composition of Black Soldier Fly Larvae

With the increasing popularity of BSF larvae as an animal feed, it is important to understand their nutritional composition. Several studies have been performed to assess how certain aspects of larval development and rearing can result in changes in the nutritional composition of the BSF. Researchers have identified that some compositional changes are inherent and will change over the course of larval development. For example, protein content is much higher in younger larvae and steadily declines as the larvae age, while dry matter (DM) and ash components increase with age (Rachmawati et al., 2010). Additionally, composition (and taste) of the larvae can be affected by the type of substrate that they are reared on. Larvae fed on swine manure have higher protein contents than those fed on cow or poultry manure (Barragan-Fonseca et al., 2017).

Therefore, all of these factors need to be considered when developing best practices for raising larvae at a commercial level for animal feed production.

2.5.1. Protein content

Protein content is one of the more important aspects to consider. Good quality fish meals have protein contents that range between 55-75%. If insect meals are to replace fish meal as a source of protein in animal feeds, then protein content should be comparable or additional ingredients may be needed to help increase the overall protein content. Unfortunately, insects typically have a lower crude protein content compared with fish. BSF larval crude protein is typically between 37-63% (DM) (Barragan-Fonseca et al., 2017). Five day old larvae have been reported to have the highest protein content within the life cycle at 61%, but these larvae only measure a few millimeters in length, have a much higher moisture content, and cannot be adequately used to breakdown waste material (Rachmawati et al., 2010). Older larvae to prepupae can be used for waste reduction and are easier to harvest, but average protein contents drop to the mid to low-40's (Newton et al., 1977; Rachmawati et al., 2010; Finke, 2013; Barragan-Fonseca et al., 2017).

The larvae's diet can significantly impact the final protein content. The highest protein contents have been measured in larvae eating liver (62.7%) and fish (57.9%) as the primary source of their diet, however these food sources are not common waste items and would likely lead to higher overall costs. More commonly used foods for BSF diets include chicken feed (47.9%), swine manure (43.4%), cow manure (42.1%), spent grains and food waste (41.7%), chicken manure (40.1%), and fresh fruits and vegetables (38.5%) (Barragan-Fonseca et al., 2017). For animal feed production and commercially

raised feeder insect industries, attention should be paid to the source of the diet and the economical trade-offs associated with higher protein contents.

Amino acid profiles should also be evaluated prior to including BSF into any diet. Previous studies have indicated that the amino acid profile for BSF larvae compares favorably with expected values for animal feeds (Newton et al., 1977). For the most part, BSF larvae have a similar profile to soybean meal, another highly utilized source of protein for animal feeds. For mammals, the rate limiting amino acids are usually those that contain sulfur side groups (e.g., cysteine and methionine). The National Resource Council determined that laboratory rats have a combined minimum requirement for cysteine and methionine of 2.3 g/kg of diet on an as is basis (NRC, 1995). Whether these requirements hold true for all animals, especially those that fall outside of the Class Mammalia, is yet to be determined. BSF larvae have a combined cysteine and methionine concentration of 3.39 g/kg on an as is basis (cysteine, 1.02 g/kg; methionine, 3.37 g/kg). This should be sufficient for most animals, although Makkar et al. (2014) recommend adding more of these amino acids to diets being offered to pigs. For birds, reptiles, and other uricotelic animals, arginine may also be an important rate limiting amino acid (Finke, 2013). Animals that undergo a complete urea cycle are able to synthesize arginine *de novo*. However, uricotelic animals do not synthesize arginine during the production of uric acid, meaning that arginine must be obtained exclusively from the diet (Inês et al., 2010). In 2013, Finke reported the arginine content of BSF larvae to be 12.3 g/kg as is, compared to the known requirement for rats at 4.3 g/kg as is (NRC, 1995). Whether this value is sufficient for a uricotelic species remains to be determined. High concentrations of dietary lysine can make matters worse, due to an

antagonistic relationship between these two amino acids (Inês et al., 2010). BSF larvae are considered to be particularly rich in lysine, and this amino acid represents 6-8% of the total protein content (Sheppard et al., 2008). Therefore, arginine content should be closely evaluated to prevent any deficiencies. Taurine is the only other amino acid concern with BSF larvae. Initial research found it to not be detectable in BSF larvae. This is of significance for mammals, but can be easily remedied by adding taurine to the complete feed. The significance of this to reptilian insectivores is currently not known (Finke, 2013).

2.5.2. Chitin content

Most protein determinations are based on the amount of nitrogen present in a food item. Since nitrogen is not commonly found in other nutrients and the amount of nitrogen within a single protein is relatively the same, a simple formula (%nitrogen x 6.25= %crude protein) can be used to estimate the amount of protein present in a food item. Unfortunately, invertebrates present a challenge for estimating protein due to the presence of chitin. Chitin is a complex nitrogen-containing molecule that closely resembles cellulose in its structure (Caligiani et al., 2018; Finke, 2007). It is well known as being an important component of an insect's exoskeleton and provides much of the exoskeleton's strength and durability. Chitin determination, however, can be a challenge. Several methods have been used to estimate the amount of chitin present within an insect. Values for chitin estimation in BSF larvae range from <1-9% DM, with most studies averaging around 5-7% (Caligiani et al., 2018; Finke, 2007; Finke, 2013; Spranghers et al., 2017). The various methodologies for calculating chitin content can make a significant impact on the adjusted protein value, thus researchers should come to a

consensus about how to best determine chitin and protein estimations. Regardless of the methodology used to estimate chitin percentage, BSF larvae are comparable to other insects in their chitin concentration. Finke (2007) estimated the chitin content of several different insects and found an average chitin content of 19.7 mg/kg as is. In 2013, using the same methodology to estimate chitin, he determined that BSF larvae were 21 mg/kg as is, a value very similar to that of domestic crickets (Finke, 2013). Since the degree of chitin within the exoskeleton can play a big role in its digestibility, BSF larvae digestibility should be considered to be similar to that for a cricket.

2.5.3. Fat content and metabolizable energy

Crude fat content of a BSF larvae can vary significantly based on diet substrate. The published range for crude fat in BSF larvae is 7-39% DM. Fresh fruits and vegetables contributed to larvae having the lowest fat content (6.63%), followed by chicken feed (14.6%), liver (25.1%), swine manure (26.4%), chicken manure (27.9%), fish (34.6%), cattle manure (34.8%), and restaurant waste (39.2%) (Barragan-Fonseca et al., 2013). Fat content can also be reduced during BSF meal production. The defatting process can be performed either by mechanical or chemical means and allows food producers to fine tune protein and fat contents separately. Any removed fat can be used in the production of biodiesel (Schiavone et al., 2017).

Crude fat values can be further refined into specific fatty acid profiles. Finke (2013) determined that 72% of the fatty acid profile was comprised of saturated fats, with lauric acid being the most prolific (51.2% as is). He also found that the larvae were able to meet NRC requirements in regards to linoleic (16.9%) and linolenic (0.65%) acids, but these values are known to be much higher in fish meal, so additional supplementation

may be beneficial. As has been stated for other nutrients, the rearing substrate can have a direct impact on the final fatty acid profile of the larvae.

As for metabolizable energy, larval insects tend to have a higher calorie content than adult insects or nymphal forms of hemimetabolous insects such as crickets (Finke, 2002). The same is true for BSF larvae. On a dry matter basis, BSF larvae were measured as being 5,139 kcal/gram (Finke, 2013). As part of a complete feed, this can be easily managed by defatted meal or just by dilution with other lower fat, plant-based ingredients. For animals that eat the insects whole, such as reptiles, this represents a very energy-dense food source and should be used in conjunction with other lower fat insects.

2.5.4. Ash content

The ash content of BSF larvae is high compared to other insects. Finke (2013) reports the ash content as 35 g/kg as is, while other popular feeder insects tend to not surpass 12 g/kg as is (Finke, 2002). The ash content is composed of the inorganic materials left behind after organic molecules are burned away. Most of that inorganic material is composed of minerals. As previously mentioned, the calcium content of the larvae is extremely high. This is due to calcium carbonate impregnated into the exoskeleton matrix (Johannsen, 1922). Although it has not been proven, the addition of this mineral into the matrix may be a contributing factor to the poor cuticular digestibility that has been encountered with this species. It is possible that calcium carbonate prevents chitinase from being able to bind to and breakdown the chitin. Other minerals, such as magnesium, potassium, iron, manganese, and zinc, are also well represented in the BSF larvae nutritional profile and contribute to the high ash content (Finke, 2013; Dierenfeld

and King, 2008). The only mineral with values below average compared to most feeder insects is sodium.

2.5.5. Vitamin content

The vitamin profile of BSF larvae is similar to that of other common feeder insects (Finke, 2013). Water soluble vitamins, including most of the B vitamins, are adequately represented to meet the requirements of the laboratory rat (NRC, 1995; Finke, 2013). Only B_6 (pyridoxine) and B_{12} come close to not meeting the requirements (Finke, 2013). Unfortunately, most insects are deficient in the fat soluble vitamins, A, D, and E (vitamin K is rarely measured). BSF larvae are no exception, with vitamins A (retinol), D_3 (cholecalciferol), and E (α-tocopherol) all reported as less than 50% of the NRC requirements (Finke, 2013). With values this low, it is imperative that these nutrients be supplied by other means. In a complete diet formulation, these may be present in high enough quantities in other ingredients. If not, they can easily be added to the diet as a supplement. Whole larvae should receive either multi-vitamin dusting or a gut loading diet with concentrations that are high enough to produce these values in the larvae before being fed to the insectivore.

2.6. Black Soldier Fly Larvae Used in Animal Feeds

BSF larvae can be utilized for animal feeds as either whole insects (live or dehydrated) or ground into a meal for ingredient use in complete feeds. Complete feeds are the preferred diet method for most of the production animal industries. Thus far, BSF meal has been well studied as an ingredient in swine, poultry, and aquaculture feeds and has been shown to produce similar health and growth parameters as animals being fed a more traditional diet (Newton et al., 1977; Bondari and Sheppard, 1981; De Marco et al.,

2015; Maurer et al., 2016, Lock et al., 2016; Renna et al., 2017; Schiavone et al., 2017; Wallace et al., 2017; Dabbou et al., 2018). In 2016, Maurer et al. compared three complete feeds for laying hens (*Gallus domesticus*). The control diet contained soybean cake as the main protein source, while the two experimental diets used partially defatted BSF meal to replace either 50 or 100% of the soybean cake used in the control diet. After three weeks of feeding, the researchers found no significant differences in body weight, egg weight, or laying performance between any of the diet groups. Similarly, Lock et al. (2016) found that Atlantic salmon (*Salmo salar*) fed diets containing BSF meal as a partial or full replacement for fish meal (25, 50, and 100% fish meal replacement) had similar weight gains as those fed the control diet after 105 days of feeding. However, these findings were only true for one type of BSF meal used. A second BSF meal product made from larvae reared on different substrates and undergoing a different processing method yielded less favorable growth results. The diets containing the second BSF meal led to lower weight gains; this was attributed to lower palatability and feed intake. Several other aquaculture studies have been performed on various fish species, with a majority of the results showing no significant differences in weight gain as long as BSF meal inclusion did not exceed 33%. Palatability and lower feed intake were cited as being the biggest factors contributing to these results (Barragan-Fonseca et al., 2017).

In addition to growth data, several studies have also looked at measures of digestibility and blood work values as additional methods of confirming the nutritive value of the food. Digestibility studies have been performed on a number of different animal species (swine, poultry, fish, cat, and dog) as a way to make general comparisons

between diets. However, differences will exist due to chemical and physical compositions of the various diets, food processing techniques, and the digestive physiologies and feed intake of the various animal species (Khan et al., 2003). In general, these studies have found that dry matter digestibility for a BSF meal complete feed to be between 59-84% (Bosch et al., 2014; Newton et al., 1977; Renna et al., 2017; Schiavone et al., 2017). Although some of these studies did find significant results when directly comparing two or more diets to each other, this range is not considered out of the ordinary for traditional diets and more research is needed to determine if the different digestibilities have any clinical significance (Bosch et al., 2014; Guimarães et al., 2012; Newton et al., 1977; Renna et al., 2017; Schiavone et al., 2017; Wallace et al., 2017). Similar acceptable digestibility coefficients have been reported for crude protein (62-91%) and crude fat (71-99%) (Bosch et al., 2014; Newton et al., 1977; Renna et al., 2017; Schiavone et al., 2017; Wallace et al., 2017). Only the swine study by Newton et al. reported digestibility data for inorganic feed components, with an ash digestibility of 45% and a soybean meal comparison at 61%. The difference between these two means was statistically significant, but there did not appear to be any clinical significance.

Only recently have researchers started looking at blood values to determine if there are differences between diet groups on a more clinical level. In guinea fowl (*Numida meleagris*) keets (0-8 weeks of age) fed graded BSF meal diets versus a fish meal control diet, the only significant changes were associated with higher lymphocyte counts in the BSF meal diets and higher triglyceride levels for most of the BSF meal diets. Overall, these birds gained more weight on BSF meal diets and no health concerns were noted for any of the birds with the higher lymphocyte counts. The researchers

suggested that despite the minor differences, both diet proteins were capable of producing healthy birds (Wallace et al., 2017).

For laying hens (24-45 weeks of age) fed either a soybean meal or BSF meal diet, the following differences were seen for the BSF meal group: higher globulin levels, lower albumin to globulin ratio, lower cholesterol and triglyceride levels, higher calcium levels, lower chloride levels, and lower creatinine levels. The lower albumin to globulin ratio, as well as the lower cholesterol, triglyceride, and creatinine concentrations, were thought to be contributed to the presence of chitin in the BSF diet as these have previously been shown to have effects on these values. The higher calcium concentration was not thought to be related to calcium in the diets, as they were similar. Instead, the difference may have been associated with higher egg laying performance for the group on the soybean diet and greater use of the body calcium stores. Overall, both diets were considered appropriate to maintain health, but that laying hens may be more productive on a soybean meal diet (Marono et al., 2017).

Lastly, Dabbou et al. (2018) looked at broiler chickens (0-5 weeks) being fed graded levels of partially defatted BSF meal (0-15% of diet, as fed basis). Maize and soybean meal made up the remainder of the protein needs. In this study, increasing BSF meal in the diet corresponded with decreasing values for triglycerides, but the differences were just shy of being classified as significant. The only significant difference seen was increased levels of phosphorous in the BSF diets, which were attributed to increased bioavailability of phosphorous from animal proteins as compared to plant sources. Overall, there were no significant changes associated with bloodwork during this short, 5 week feeding study. This study did also evaluate the effects of diet on organs by

incorporating histopathology. For most organs, there were no differences in the pathology score. However, within the intestines, birds that received higher percentages of the BSF meal in their diet had significantly shorter intestinal villi and deeper crypts. These changes are often associated with negative gut development, reduced absorption of nutrients, higher cell turnover, and poorer performance. There were zero mortalities and no signs of illness in these birds during the course of the study, indicating that the diets were not causing any changes that were significant enough to cause disease within this short time period. The researchers suggested that the improved growth during the starter period and the absence of any clinical signs or hematological/biochemical abnormalities were enough evidence to include small quantities (5-10% of diet) of BSF meal in broiler chicken feeds.

2.7. Black Soldier Fly Larvae Used as Feed for Exotic Species

Most of the research that has been performed on BSF larvae over the years has been focused on livestock and aquaculture production. To date, only two studies have evaluated BSF larvae as a food source for exotic animals. In 2007, Bodri and Cole conducted an experiment that compared diets for hatchling American alligators (*Alligator mississippiensis*) in a commercial facility, similar to the experiments performed for other production species. In the wild, the diet of a hatchling alligator is primarily comprised of insects and other invertebrates (Delany, 1990). Given the expensive nature of commercial diets composed of fish meal, Bodri and Cole investigated the differences in growth rates for alligators fed a more natural BSF diet (whole, dried larvae) versus two commercial diets that varied in protein content (56% and 45% protein). The hatchlings were offered the diets exclusively for three months. Morphometric measurements were

then compared between groups. The commercial diet with the highest protein content produced the heaviest and longest alligators, as was expected. A growth disparity was still seen between the remaining two groups, even though the second commercial diet and the whole BSF larvae had similar protein contents. The 45% protein commercial diet produced alligators with a median weight of 93.1 grams and a snout-to-vent length (SVL) of 14.3 cm, while the BSF larvae group produced alligators with a median weight of 59.0 grams and SVL of 13.8 cm.

Palatability and size of the food items were thought to be the most likely explanations for the differences in growth. Hatchlings fed the pelleted diets would start feeding immediately and there was minimal waste. Researchers also commented that the round shape of the pellets appeared to be easier for the hatchlings to grasp. For the BSF diet, the alligators showed little interest in the diet and the bigger, flattened shape of the larvae may have been more difficult to prehend. The obvious differences in food intake explain the differences in growth over the three-month time period. However, based on the amount of growth that occurred while ingesting such small quantities of food, it did appear that the BSF larvae had a superior feed conversion rate. The researchers conjectured that had the BSF group ingested similar amounts of larvae as the pelleted groups, that they would have grown as much as, if not more, than those eating the commercial diets.

Unlike many of the experiments performed with livestock and aquaculture, this study did not account for other nutritional differences between the diets. The commercial diets were complete feeds with balanced levels of vitamins and minerals, while the BSF larvae are not balanced and devoid of fat soluble vitamins. A better comparison would

have been to use BSF meal as a protein substitution in the pelleted diet or to gut load the insects prior to drying to reach comparable levels to the other diets. Additionally, measuring other indicators of the food's nutritive value, such as digestibility or plasma biochemistry concentrations of the alligators, would have made for a stronger evaluation of the diet.

In 2008, Dierenfeld and King were the first researchers to assess BSF larvae outside of a production system. After feeding a collection of frogs BSF larvae, they made the observation that whole larvae were being passed intact in the fecal material. This suggested that the frogs were unable to digest any of the nutrients within the larvae. Using mountain chicken frogs (*Leptodactylus fallux*) as the model species, Dierenfeld and King fed the frogs three different diets and measured apparent digestibility for each. The frogs were fed calcium dusted crickets, live BSF larvae, and mashed BSF larvae through a feeding tube. Feces were collected during each diet phase and analyzed for nutrient content. Their results showed that the frogs were not able to adequately digest the live BSF larvae. Dry matter digestibility for crickets and mashed larvae were similar at 86% and 76%, respectively. Live larvae on the other hand were only 26% digestible. Overall, live BSF larvae were less digestible than crickets for every single nutrient that was measured. For calcium, only 44% was digested compared to 84% for dusted crickets. When the BSF larvae were mashed, calcium digestibility increased to 88%. The high calcium content of the larvae is the main selling point for use in reptiles and amphibians. If the calcium is poorly digestible, the larvae will still require calcium dusting or gut loading to meet the consumer's physiological requirements. Previous literature has identified that the larvae's high calcium content is due to calcium carbonate

impregnated within the exoskeleton matrix (Johannsen, 1922). When the exoskeleton is disrupted, such as occurred with the mashed diet, the calcium, as well as other nutrients, become more available to the consumer. The authors' theorized that animals with an alternative feeding strategy, chewing prey versus swallowing whole (as is the case with many frog species), may cause enough disruption to the exoskeleton to improve digestibility.

Unfortunately, Dierenfeld and King also identified another potential issue for using these larvae as a main diet staple. The live larvae actually produced negative digestibilities for fiber, sodium, copper, iron, and molybdenum, indicating a loss of these body nutrients. When the larvae were mashed, these nutrients became much more bioavailable, with the exception of sodium. Sodium for the mashed larvae still maintained a negative digestibility (-489%), and the authors were concerned that the larvae may be inducing gut irritation and a "diarrhea-like" syndrome. No other studies have looked at digestibility of minerals when feeding the larvae, so whether this syndrome is widespread across species is unknown. They suggested performing long term studies to track potential fecal losses and to perform plasma biochemistries to assess animals for changes in electrolytes when feeding the larvae on a long term basis. Additionally, histopathology of the intestines, such as was performed for the broiler chickens previously, may help to determine whether these sodium losses are truly associated with gut irritation and villi shortening (Dabbou et al., 2018).

2.8. Research Objectives

Most of the research evaluating BSF larvae in the diet of animals suggests that these insect can be used to maintain growth and promote reproduction. However, there

does appear to be a few issues that could limit their usefulness as a feeder insect or as a component of a diet for reptiles, especially when fed over a long period of time. The first objective of this thesis project was to determine if BSF larvae could be gut loaded in a predictable manner to compensate for its nutritional deficiencies, particularly for fat soluble vitamin A. The second objective was to measure the digestibility of the larvae in a common insectivorous reptile species, the leopard gecko (*Eublepharis macularius*), and determine if target nutrients (calcium, vitamin A) could be absorbed in sufficient quantities by the target species without the use of dusting powders. Additionally, we wanted to know whether simple disruption of the cuticle (piercing with a needle) would improve the issue of digestibility. The third and final objective was to determine whether BSF meal could be used in a carnivorous reptile, the corn snake (*Pantherophis guttatus*), to maintain health and growth of the animal.

CHAPTER 3.
EVALUATION OF VITAMIN A GUT LOADING IN BLACK SOLDIER FLY LARVAE (*HERMETIA ILLUCENS*)

3.1. Introduction

Nutritional disorders are a common problem observed in captive reptiles and amphibians, especially for insectivorous species (Mans and Braun, 2014; Latney and Clayton, 2014). Nutritional secondary hyperparathyroidism and hypovitaminosis A are two of the most common nutritional disorders being managed in a clinical setting (Mans and Braun, 2014). Nutritional analyses have been performed on a majority of the insects commercially raised for food, and while these analyses have shown that insects provide an excellent source of most nutrients, they are usually deficient in calcium, fat soluble vitamins (A, D, and E), thiamine, and omega 3-fatty acids (Finke, 2002; Finke, 2003; Finke, 2013). To correct these deficiencies, veterinarians and herpetoculturists have employed two strategies to improve the insect's nutritional quality: dusting and gut loading. Dusting insects with calcium and multi-vitamin powders is simple and can be done immediately prior to the insects being offered as food; however, this method has been shown to be unreliable as over 50% of the dust falls off or is groomed away in under 2.5 minutes when applied to crickets (Li et al., 2009). Gut loading insects with calcium and vitamin rich diets is preferred (Allen, 1997); however, these diets can provide variable results based on the duration of gut loading, developmental cycle of the insect, nutritional aspects and quality of the gut loading diet, decreased palatability of the diet to the insect, and higher insect mortality due to nutritional toxicosis (Finke et al., 2005; Livingston et al., 2014). Because of these limitations, finding insect prey that are more nutritionally complete is warranted.

Black soldier fly (BSF) larvae (*Hermetia illucens*) are a unique feeder insect because they have a mineralized, calcium carbonate exoskeleton, making them highly calcium rich (Johannsen, 1922). The calcium to phosphorus ratio for a fasted BSF larvae is approximately 2.5:1, which is significantly higher than other popular feeder insects such as common house crickets (*Acheta domesticus*), at 1:7, and mealworms (*Tenebrio molitor*), at 1:17 (Dierenfeld and King 2008; Finke, 2002; Finke, 2013). While these insects are mineral dense, they are, unfortunately, still deficient in fat soluble vitamins. True vitamin requirements are currently unknown for reptiles and amphibians, but recommendations are based on the known requirements in other species. In a 2013 study published by Finke, it was found that the concentrations of vitamins A, D, and E in fasted BSF larvae were all below the National Resource Council's (NRC) requirements for growth in laboratory rats and poultry. Vitamin A was less than 50% of this requirement (NRC, 1995; NRC, 1996). Given vitamin A's importance for growth, reproduction, and immunity, further research into providing adequate supplementation is necessary.

Multiple studies have been performed assessing the efficacy of gut loading vitamin A into crickets and mealworms, but there is currently no published data regarding the efficacy or feasibility of gut loading BSF larvae for food use in insectivore species (Allen, 1997; Attard, 2013; Finke, 2003; Finke et al., 2005; Hunt Coslik et al., 2009; Li et al., 2009; Livingston et al., 2014). Although black soldier fly larvae are a relatively new addition to the exotic pet food market, they have been well studied in the fields of entomology and livestock production (Barragan-Fonseca et al, 2017; Bondari and Sheppard, 1981; Cammack and Tomberlin, 2017; Diener et al., 2009; Hale, 1973; Holmes et al., 2016; Newton et al., 1977; Sheppard et al., 2002; Tomberlin et al., 2009;

Tomberlin et al., 2002). These previous studies have identified several biotic and abiotic factors associated with rearing BSF larvae that are likely to affect the gut loading process (Diener et al., 2009; Holmes et al., 2016; Tomberlin et al., 2002; Tomberlin et al., 2009). The primary objective of this study was to identify factors associated with gut loading vitamin A into BSF larvae and to develop feeding recommendations for a more consistent gut loading process. Factors that were addressed included the vitamin A concentration added to the diet, length of time given to gut load, moisture content of the diet, and density of larvae during feeding. We hypothesized that larval vitamin A concentrations would increase in a predictable manner with increasing levels of dietary vitamin A given that gut loading and environmental conditions were held constant. Additionally, we hypothesized that BSF larvae would have a higher vitamin A concentration if they were given a shorter gut loading time period of 24 hours, a higher substrate moisture content, and a lower larval density.

3.2. Materials and Methods

3.2.1. Animals

BSF larvae were obtained from Fluker Farms, Inc. (Port Allen, LA) at the 4th or 5th instar (size large). Larvae had been chilled and fasted for at least one day prior to the start of the gut loading experiments.

3.2.2. Diet and environmental conditions

The base diet fed to all BSF larvae during the course of the experiment was a mixture of 65% wheat bran and 35% corn meal, a modification of the Gainesville fly larvae diet currently recommended for raising BSF (Cammack and Tomberlin, 2017;

Sheppard et al., 2002; Tomberlin et al., 2002). All experiments were conducted at ambient room temperature and relative humidity (22-25°C, 30-40% RH).

Vitamin A concentration and gut load time experiment: Five different vitamin A concentrations were tested to determine the larval diet levels needed to achieve appropriate gut/tissue concentrations and to assess larvae for signs of toxicosis or changes in appetite based on the various vitamin concentrations provided. A water-soluble vitamin A solution (Rovimix A 500-WS [150,000 µg/g]; DSM Nutritional Products, Ames, IA) was diluted to 300 µg/mL and a calculated amount was added to each diet to reach the experimental concentrations of 0; 5,000; 10,000; 15,000; and 20,000 µg/kg of diet on a dry matter basis (DMB). Additional water was added to each diet to reach an approximate final moisture content of 60% and then mixed well. For each diet concentration, three separate containers were prepared individually using covered, five-gallon buckets. Each bucket consisted of one kilogram of modified Gainesville diet on a wet matter basis and approximately 1000 larvae that were allowed to gut load over a 24-hour time period. Forty-eight hour samples were also collected from the 15,000 and 20,000 µg/kg groups to better assess length of time that should be allowed for gut loading. Two samples of diet (50 g each) were removed from each bucket at the beginning of the experiment and two samples of larvae from each time point (25 g each) were collected from each bucket and held in a -80°C freezer until they could be analyzed for vitamin A concentration using high performance liquid chromatography (HPLC; SDK Laboratories, Hutchinson, KS).

3.2.3. Substrate moisture experiments

Fifty larvae were added to clear, circular, plastic containers (dimensions 8.9 cm diameter x 14 cm height) each containing 10 grams of modified Gainesville diet on a dry matter basis. Increasing volumes of water (0, 5, 10, 13.75, 17, and 40 ml's) were added to the base diet to create increasing levels of substrate moisture (8%, 40%, 56%, 60%, 65%, and 77%, respectively). Percent moisture was determined using a rapid moisture analyzer (Torbal BTS110, Scientific Industries, Inc., Bohemia, NY). Collective weights of the larvae were obtained prior to addition to the diet and after removal from the diet at 24 hours. All larvae were dried completely prior to obtaining the final weights. Changes in weight over time were used to assess how well the larvae fed in the moisture conditions provided. Two replicates were performed for each substrate moisture condition.

A second substrate moisture experiment was performed that assessed the differences in larval vitamin A concentrations when allowed to gut load on two diets of differing substrate moistures. One kilogram of modified Gainesville diet was used for each and a water-soluble formulation of vitamin A (Rovimix A) was added to each diet to reach an expected 20,000 µg/kg of diet on a dry matter basis. Two thousand large BSF larvae were added to each diet once prepared and kept in a covered, five-gallon bucket for a 24-hour gut loading period. For diet 1, the vitamin A formulation was first diluted in water to 300 µg/ml. The diluted vitamin A solution was then sprayed over the diet to achieve even distribution and mixed well. Diet 1 was then allowed to dry in a conventional oven for 30 minutes at a temperature of 70°C, mixed again halfway through, and left overnight to cool before adding larvae to the diet. For diet 2, the

vitamin A was diluted in 1,250 mL of water reaching a final concentration of 16 µg/ml. The vitamin A dilution was poured directly onto the diet, mixed well, and larvae were added immediately. The moisture contents of the diets were measured using the rapid moisture analyzer just prior to addition of the larvae. Diets 1 and 2 were measured as having a moisture content of 8% and 60%, respectively. Three representative samples from each diet and each larvae group were collected and frozen at -80°C until they could be further processed. Each sample was then analyzed for vitamin A content using HPLC (SDK Laboratories, Hutchinson, KS).

3.2.4. Larval density experiment

Either 25 or 50 larvae were added to clear, circular, plastic containers containing various increments of the modified Gainesville diet with water added to achieve 65% moisture content. The experimental larval densities were as follows: 0.1, 0.5, 1, 5, 10, 15, and 25 larvae per gram of wet diet. Collective weights of the larvae were collected prior to addition to the diet and after removal from the diet at 24 hours. Changes in weight over time on an average per larvae basis were used to assess how well the larvae gut loaded in the density conditions provided. Two replicates were performed for each density condition.

3.2.5. Statistical Analysis

Distributions of the data were evaluated by use of the Shapiro-Wilk test, skewness, kurtosis, and q-q plots. Data that were not normally distributed were logarithmically transformed in order to perform parametric analysis. Linear regression was used to determine whether the concentrations of vitamin A added to the diets were predictive of final larval vitamin A concentrations. A repeated measures ANOVA was

used to assess the differences in larval vitamin A concentrations between the 24 and 48 hour gut loading time periods. A one-way ANOVA was used to evaluate the changes in weight for larvae fed under different moisture and density conditions. An independent samples t-test was used to determine if a difference existed between vitamin A concentrations of larvae fed at two different substrate moistures. All statistics were performed and analyzed using SPSS 25.0 (IBM Statistics, Armonk, NY). A value of $p \leq 0.05$ was used to determine statistical significance.

3.3. Results

3.3.1. Vitamin A concentration and gut load time experiment

The BSF larvae were fed five experimental diets with varying concentrations of vitamin A (391-20,108 µg/kg DMB). The effect of dietary vitamin A concentration on the larval vitamin A concentration after 24 and 48 hours can be seen in Table 3.1. A non-linear regression line is observed in Figure 3.1 and is best described by a second-order polynomial equation ($y=3E\text{-}06x^2 – 0.0086x + 39.715$; $r^2=0.9939$). For the 15,000 and 20,000 µg/kg diets, final larval vitamin A concentrations between diet groups and the length of time allowed for gut loading were evaluated. A significant difference was seen between the two diet groups with larvae feeding on the 20,000 µg/kg diet having higher vitamin A concentrations at both time periods ($F=10.696$, $p=0.017$). Also, larvae fed for 24 hours were significantly more likely to have higher vitamin A concentrations than those fed for 48 hours regardless of diet concentration ($F=150.818$, $p=0.000$).

3.3.2. Substrate moisture

The average change in larval weight over 24 hours of gut loading was measured for various substrate moistures (Table 3.2). A significant weight gain was seen for larvae

Table 3.1. Larval vitamin A concentrations based on diet concentration and time. Five diets with increasing vitamin A concentrations and the resulting BSF larval vitamin A concentrations after 24 and 48 hours of gut loading (Mean ± SD, n=6 for each diet group, n=6 for each larval group).

Diet Formulation	Measured Diet Vitamin A Concentrations (µg/kg DMB)	Larvae Vitamin A Concentrations at 24 hours (µg/kg as fed)	Larvae Vitamin A Concentrations at 48 hours (µg/kg as fed)
Control: 0 µg/kg	391 ± 40	17 ± 11	--
5,000 µg/kg	4,532 ± 828	91 ± 22	--
10,000 µg/kg	8,932 ± 927	235 ± 40	--
15,000 µg/kg	15,850 ± 1,683	637 ± 125[a]	242 ± 24[b]
20,000 µg/kg	20,108 ± 798	1,167 ± 169[a]	374 ± 69[b]

[a,b]= Significantly different values within the same row are indicated by a different letter

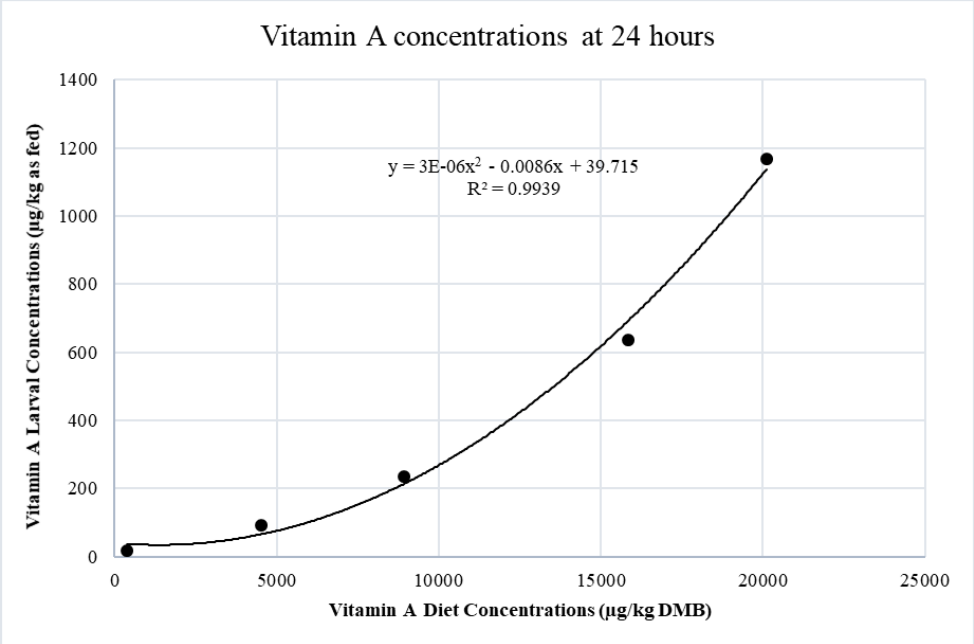

Figure 3.1. Effect of dietary vitamin A on BSF larval vitamin A concentrations after 24 hours of gut loading.

feeding on substrates with 56-77% moisture (F=41.436, p=0.000). Minimal weight gain or loss of weight was seen in larvae that were offered substrates with lesser moisture contents. To verify that weight gain was not just associated with heavier diets within the gastrointestinal tract, two diets consisting of approximately 20,000 µg/kg of vitamin A each were also evaluated over the course of 24 hours of gut loading (Diet 1= 8% moisture content, Diet 2= 60% moisture content). The average larval vitamin A concentration for Diet 1 was 858 ± 0 µg/kg on an as fed basis. This was compared to an average of 1,352 ± 100 µg/kg on as fed basis for larvae being offered Diet 2 (t=-8.498, p=0.001) (Table 3.3).

Table 3.2. Effect of substrate moisture on larval feeding and weight changes over the course of 24 hours. (Mean ± SD, n=2 per group)

Substrate moisture content	Average change in weight per larvae
0 ml added = 8%	-0.005 ± 0.001 g[a]
5 ml added = 40%	-0.001 ± 0.006 g[a]
10 ml added = 56%	0.024 ± 0.001 g[b]
13.75 ml added = 60%	0.021 ± 0.002 g[b]
17 ml added = 65%	0.026 ± 0.001 g[b]
40 ml added = 77%	0.026 ± 0.003 g[b]

[a,b]= Same letters indicate values within the column that are not significantly different from each other using a Bonferroni post-hoc analysis. Different letters within the column indicate a significant difference.

3.3.3. Larval density

The average change in larval weight over 24 hours of gut loading was also measured for various larval density conditions (Table 3.4). A significant weight gain was

seen for larvae housed between the densities of 0.1-1 larvae per gram of wet substrate (F=78.407, p=0.000). Larvae housed within these densities gained on average 0.021 ± 0.002 grams as compared to an average weight gain of 0.009 ± 0.003 grams for the remaining density groups (5, 10, 15, and 25 larvae/g of diet).

Table 3.3. Effect of substrate moisture on larval gut loading and final vitamin A concentrations. (Mean ± SD, n=3 for each diet and larval vitamin A measurement).

Diet Type	Diet Vitamin A content (µg/kg DMB)	Larval vitamin A content (µg/kg as fed)
Diet 1 (8% moisture)	31,779 ± 6,319	858 ± 0[a]
Diet 2 (60% moisture)	21,177 ± 1,164	1,352 ± 100[b]

[a,b]= Different letters indicate a significant difference between the two groups of larvae.

Table 3.4. Effect of larval density on larval feeding and weight changes over the course of 24 hours. (Mean ± SD, n=2 for each group).

Number of larvae per gram of wet diet	Average change in weight per larvae
0.1	0.022 ± 0.002 g[a]
0.5	0.019 ± 0.001 g[a]
1	0.021 ± 0.000 g[a]
5	0.011 ± 0.001 g[b]
10	0.011 ± 0.001 g[b]
15	0.008 ± 0.001 g[b,c]
25	0.005 ± 0.001 g[c]

[a,b,c]= Same letters indicate values within the column that are not significantly different from each other using a Bonferroni post-hoc analysis. Different letters within the column indicate a significant difference.

3.4. Discussion

The results of these experiments show that BSF larvae can be gut loaded with vitamin A in a predictable manner, similar to what has been published for crickets and

mealworms, as long as they are kept under a consistent set of conditions (Finke, 2003). Temperature, relative humidity, diet composition, substrate moisture, larval density, light exposure, and light intensity have all been shown to have significant impacts on the rearing of black soldier flies (Barragan-Fonseca et al., 2017; Cammack and Tomberlin, 2017; Diener et al., 2009; Holmes et al., 2016; Sheppard et al., 2002; Tomberlin et al., 2002; Tomberlin et al., 2009). For the purpose of this experiment, we chose to focus on the conditions that could be easily manipulated and that would likely have the biggest impact for feeding over the course of a short period of time.

The ideal temperature for rearing BSF larvae has been published to be 26-27°C (79-81°F); however, these insects are capable of being raised in temperatures ranging from 12-36°C (54-97°F) (Barragan-Fonseca et al., 2017; Holmes et al., 2016; Sheppard et al., 2002; Tomberlin et al., 2009). For this experiment, we chose to maintain our gut loading larvae at room temperature, which across the course of all experiments, ranged from 22-25°C (72-77°F). The reason behind this decision was to try and mimic the conditions that most institutions and owners would be maintaining for their own colonies. The inconsistency in temperature across the experiments, undoubtedly, resulted in some variability with the gut loading process, but the authors believe that this variance is acceptable and will likely be experienced by others trying to gut load larvae for their own animals. Following this same idea, relative humidity was also maintained at normal room levels (30-40%). The literature lists the ideal humidity for rearing to be between 60-70%, with an acceptable range reported as being 25-99% (Barragan-Fonseca et al., 2017). However, in our efforts to mimic the conditions that would allow for the easiest

maintenance of a colony, we chose to use normal indoor room conditions versus those that have been published as being ideal.

Diet composition is one of the more extensively studied subjects for BSF larvae rearing and production. As generalist decomposers, they are capable of being raised on several different substrates including layer hen rations, post-consumer food wastes, and even animal feces. Based on the diet they receive, their nutritional profile can be altered (Barragan-Fonseca et al., 2017; Cammack and Tomberlin, 2017; Sheppard et al., 2002; Tomberlin et al., 2002). The type of diet may also change the larvae's rate of feeding, introducing a much higher degree of variability to the gut loading process. For this reason, an inexpensive and easily replicated diet, that still offered a good nutritional profile, was chosen for all experiments (Cammack and Tomberlin, 2017; Sheppard et al., 2002; Tomberlin et al., 2002).

Of the remaining conditions, light exposure and intensity were not expected to make any substantial change over the course of a short period of time and have been found to be more important factors for mating rather than larval growth (Barragan-Fonseca et al., 2017). Substrate moisture and larval density, on the other hand, were considered to be easily manipulated and potential factors that could affect feeding over a short period of time. The results obtained from these experiments do indicate that these conditions are very important to consider when gut loading BSF larvae. Unlike the mealworm that is usually fed a dry grain substrate, BSF larvae prefer a moist diet substrate (Tomberlin et al., 2002). Here, we found that BSF larvae fed diets with moisture levels between 56-77% appeared to feed better than those fed on lower moisture diets. This coincides with previous research that has suggested that substrate moistures

should be between 52-70% (Barragan-Fonseca et al., 2017). Since higher moisture foods are heavier, it is possible that larvae consuming the wetter diets only weighed more due to the higher water content. However, based on some of our unpublished pilot data and the second substrate moisture experiment that confirmed higher vitamin A concentrations in larvae fed a diet at 60% moisture compared to 8%, we do know that an actual difference exists between feeding rates. The exact range of substrate moistures capable of yielding the highest vitamin A concentrations is not known at this time and could be further studied, but it is likely close to the range we reported for the first experiment. It is important to note that the 77% moisture diet did produce heavier larvae; however, it is possible that the increased weight gain could have been attributed to water weight and less attributed to vitamin A covered food within the gastrointestinal tract. As more water is added to a diet, the gut loaded nutrients are likely to see a dilutional effect, similar to crickets that have been offered higher moisture foods (e.g., potatoes and lettuce) during a gut load period (Livingston et al., 2014). Additionally, the more moisture present in the diet, the harder it is to remove the larvae with simple sifting. For the purposes of efficiency and cleanliness, and based on our current results, the authors recommend that dietary moisture content not exceed 65% as this was the highest moisture content analyzed for final larval vitamin A concentrations.

The last condition that was tested was larval density. Previous research has indicated that 10 larvae per gram of diet yields the best trade-off between waste reduction efficiency and increased larval weight (Diener et al., 2009). Altogether, studies have looked at densities between 0.3-80 larvae per gram of diet and have found that larvae fed at lower densities have increased weights and shorter development times compared to

larvae at higher densities that must feed on and utilize more of their own waste products (Barragan-Fonseca et al., 2017; Sheppard et al., 2002). We hypothesized that we would see a significant increase in weight for lower densities, even within the short window of time needed for gut loading. Our hypothesis proved to be true with densities between 0.1-1 larvae per gram of wet food weighing significantly more than larvae maintained at higher densities. Although, larval vitamin A concentrations were not tested during the density portion of this experiment, it stands to reason that larvae ingesting more food will also have higher concentrations of vitamin A within their gastrointestinal tract.

However, increased gut loading and weight gain potentially come at a price. As was mentioned above, lower densities lead to shorter development times. On average, the black soldier fly has a larval development time of 24 days with larval molts occurring every four days, but when given ideal conditions they can develop in 16 days with molts occurring every 2.5 days (Barragan-Fonseca et al., 2017; Diener et al., 2009). Larval molting is a very important aspect to consider for gut loading due to the fact that just prior to every molt, food consumption declines and insects must "shed their gut" in order to prepare for the next larval stage (Attard, 2013; Woodring, 1983). As insects prepare to molt, the exoskeleton separates from the underlying structures and most fecal matter is expelled. "Shedding the gut" refers to the portion of exoskeleton that lines the lumen of the intestines, also known as the peritrophic membrane, that is shed with the rest of the insect's cuticle (Gullan and Cranston, 2014). Therefore, molting has a huge impact on one's ability to reliably obtain gut loaded larvae. Even with all environmental conditions held the same, larval vitamin A concentrations may drop as much as 50% due to a majority of the larvae being close to a molting stage.

The best way to reduce variability is to ensure proper timing. For all of our experiments, larvae were picked up directly from the supplier. The larvae were kept in a chiller set to 16°C (60-61°F) for at least 24 hours and were fasted during this time. They were then placed under the previously described experimental conditions to allow for gut loading over the next 24-48 hours. Feeding the larvae directly out of a chilled, fasted, and quiescent stage ensured that most of the larvae would need to eat in order to reach their next critical weight for molting to the next stage of larval development. When measuring vitamin A concentrations over the two gut loading time periods, the larvae had higher concentrations after 24 hours as opposed to 48 hours. For crickets, 48 hours has long been believed to be the ideal time period for gut loading according to years of past research (Allen, 1997; Attard, 2013; Finke, 2003; Hunt Coslik et al., 2009; Livingston et al., 2014). But for the BSF larvae, this time period appears to be too long. The lower concentrations seen at 48 hours are most likely tied to decreased consumption prior to molting. Given the short time period between molting stages for larvae kept under ideal conditions, this is not a surprising finding. The scope of this research did not investigate other time periods, but the authors believe that further investigation into even shorter time periods (4-24 hours) is well warranted. Recent evidence may even suggest that shorter gut loading time periods should be further investigated in other feeder insect species as well, especially when semi-moist diets are used compared to dry grain products (L. Molitor, personal communication, September 27, 2018).

Lastly, the authors would like to note that not all vitamin A products are equal. Within the pilot trials of this study (unpublished data), a different version of vitamin A was used that was manufactured as an oil droplet. Even though it tested as being the right

concentration, it did not mix well into the diet and led to very inconsistent concentrations. Powdered, food grade forms of retinyl palmitate have been found to be much more reliable at mixing and should be used if available (Finke, 2003). Beta carotene powders are another possible source, but will require conversion into the final vitamin A form by either the larvae or the insectivore.

3.4.1. Conclusions

Based on the information collected during this study, the authors were able to formulate the following list of recommendations for gut loading BSF larvae:

1. Larvae should be fasted and chilled for at least 24 hours to prevent the larvae from entering a molting stage prior to the end of the gut loading period. During the feeding process, the larvae should be maintained at ambient room temperature and relative humidity (22-25°C, 30-40% RH) for easier care and so as to not speed up larval development any further.
2. Water should be added to a mixture of wheat bran and corn meal to provide a consistent gut loading diet. Water should be added just prior to addition of larvae to ensure proper moisture content and to prevent mold growth. Ideally, substrate moisture should be between 60-65%, but levels as low as 56% or as high as 77% moisture may also produce similar results.
3. Larval density within the substrate should be kept between 0.1-1 larvae per gram of wet diet.
4. Larvae should be given no longer than 24 hours to gut load.
5. Vitamin A should be added to the larval diet at a concentration between 16,000-20,000 µg/kg on a dry matter basis to produce larval concentrations above 700

µg/kg (the minimum requirement published for laboratory rats) (NRC, 1995). For the most consistent results, food grade vitamin A powder (such as Rovimix-A WS) should be used. If another form is used, additional analyses should be performed to ensure proper mixture and uptake into larvae.

Although gut loading has been shown to be a variable process across multiple feeder insect species, following these recommendations should lead to greater consistency. Next steps for research should include additional time points for gut loading and further study of BSF larvae digestibility and nutrient uptake into the target consumers.

CHAPTER 4.
DIGESTIBILITY OF BLACK SOLDIER FLY LARVAE WHEN FED TO LEOPARD GECKOS (*EUBLEPHARIS MACULARIUS*)

4.1. Introduction

Black soldier fly (BSF) larvae (*Hermetia illucens*) are a popular feeder insect because they are the only commercially produced insect that has a natural positive calcium to phosphorous (Ca:P) ratio (2.5:1) (Boykin et al., 2018b; Dierenfeld and King, 2008; Finke, 2013). A positive calcium to phosphorous ratio (ideally 2:1 or greater) is considered essential to reducing the incidence of nutritional secondary hyperparathyroidism (NSHP) in insectivorous reptiles (Boyer and Scott, 2019a; Klaphake, 2010). The black soldier fly larvae's high calcium level is due to an abundance of calcium carbonate impregnated within the exoskeleton (Johannsen, 1922). However, previous studies and anecdotal reports have indicated that these larvae, in particular the calcium-rich exoskeleton, are poorly digestible and may not be capable of providing sufficient calcium to prevent disease. Dierenfeld and King (2008) looked at BSF larvae digestibility in mountain chicken frogs (*Leptodactylus fallux*) by analyzing fecal matter for nutrients and found that calcium digestibility was only 44% for frogs ingesting whole BSF larvae as compared to 84% when they were fed dusted crickets. When the BSF larvae (and exoskeleton) were mashed with a mortar and pestle, calcium digestibility increased to 88%. This suggest that physical disruption of the exoskeleton, such as would occur with mastication, could improve the calcium availability to the consumer. Dierenfeld and King theorized that species that were more apt to chew their food prior to ingestion would likely see higher levels of calcium digestibility. Producers of the larvae

have also made the recommendation that piercing the exoskeleton with a needle prior to feeding could help to improve issues with digestibility, but neither theory has been tested.

Of course, calcium is not the only nutrient that needs to be considered when constructing a diet for insectivorous species. In addition to NSHP, hypovitaminosis A is another common condition associated with insectivorous reptiles (Boyer, 2019; Mans and Braun, 2014). Most feeder insects, including BSF larvae, are severely deficient in the fat soluble vitamins (A, D_3, and E), and thus still require multivitamin dusting or gut loading (Finke, 2002; Finke, 2003; Finke, 2013, Pennino et al., 1991). Vitamin A is important for a number of different bodily functions, including growth and development, immunity, vision, reproduction, and health and function of glands, ducts, and mucous membranes (Abate et al., 2003; D'Ambrosio et al., 2011; Ferguson et al., 1996; Mans and Braun, 2014). Although true requirements for vitamins are not known for any reptile species, recommendations are currently based on the known requirements for laboratory rats (NRC, 1995; Finke, 2003). Previous research from the authors has proved the feasibility of gut loading vitamin A into BSF larvae (Boykin et al., 2018b), but determination of whether it can be digested and absorbed by a reptile requires further study.

The goal of this research was to determine if BSF larvae, given their potential issues with digestibility and vitamin deficiencies, can provide an adequate source of nutrition for leopard geckos (*Eublepharis macularius*). Leopard geckos were selected as a model because they are a common insectivorous species that are well known to be susceptible to nutritional disorders such as NSHP and hypovitaminosis A. Additionally, their smaller size makes them more apt to at least partially chew their food as compared to larger species. Our specific objectives were to determine digestibility of whole versus

needle-pierced BSF larvae in a leopard gecko model and to determine if calcium and vitamin A could be absorbed in sufficient quantities by the target species without the use of dusting powders. We hypothesized that 1) digestibility of BSF larvae would increase by piercing the exoskeleton with a needle prior to being offered to the leopard geckos, and that 2) geckos receiving vitamin A gut loaded BSF larvae would have higher liver vitamin A concentrations than those receiving non-gut loaded BSF larvae

4.2. Materials and Methods

4.2.1. Animals and husbandry

This research was conducted in accordance with the rules and regulations set by the Louisiana State University's Animal Care and Use Committee (protocol #17-083). Twenty-four male leopard geckos of unknown age were obtained from a private breeding colony with an average initial weight of 50.7 ± 11.5 grams (range 31.6-72.1 g). Males were recruited to rule out any potential bias associated with female reproduction. Each gecko was individually housed in a clear plastic terrarium (43cm x 21cm x 25cm) without substrate and maintained at 28-30°C (83-86°F) and 30-40% relative humidity. Overhead fluorescent lamps provided lighting on a 12:12 light:dark cycle. The lighting did not provide any ultraviolet B radiation. The geckos had access to hide houses and water *ad libitum*. Each gecko was started on a vitamin A depleted diet (fasted crickets and BSF larvae) during the acclimation period. Twenty of the geckos were maintained on this diet for 75 days before starting the feed trial, while four geckos underwent a 26 day acclimation period. The latter group was needed to replace four original subjects that would not eat BSF larvae.

4.2.2. Physical and ophthalmic exams

Prior to the start of the feeding experiments, each gecko underwent a full physical exam. Fecal samples were collected and no parasites were seen. Repeat physical exams were performed at the end of the study. In order to assess the geckos for ocular changes associated with hypovitaminosis A, ophthalmic exams were performed on awake geckos by a boarded veterinary ophthalmologist at the beginning and end of the study. Exams included full visual assessment of the external structures, cornea, anterior chamber, and lens, as well as rebound tonometry (Icare® Tonovet, Vantaa, Finland, using the p setting).

4.2.3. Sedation and bloodwork values

Each animal was sedated to facilitate handling for physical exams and venipuncture. Sedation was achieved by using a combination of dexmedetomidine (Dexdomitor, 0.1 mg/ml; Zoetis Services LLC, Parsippany, NJ) at 0.1 mg/kg and midazolam (1 mg/ml; West-Ward Pharmaceuticals Corp., Eatontown, NJ) at 1 mg/kg, subcutaneously in the axillae (Doss et al., 2017; Doss et al., 2018). Once the examinations were completed, the geckos were reversed with atipamezole (Antisedan, 5 mg/ml; Zoetis Services LLC, Parsippany, NJ) at 1 mg/kg and flumazenil (0.1 mg/ml, manufacturer) at 0.05 mg/kg, subcutaneously in the axillae. Due to issues with recovery in another leopard gecko study running simultaneously, the dexmedetomidine dose was decreased to 0.05 mg/kg for the Day 35 and Day 105 blood draws and flumazenil was discontinued for the remainder of the study. Sedation level remained appropriate for venipuncture with this dexmedetomidine dose.

Blood was collected via the cranial vena cava using a 3 ml syringe and 25-gauge needle. Samples were placed in a lithium heparin microtainer, centrifuged within 30 minutes of collection, and chilled on ice. Within three hours of collection, all plasma was separated and frozen at -80°C until further processing could be performed. Two blood samples were obtained during the acclimation/vitamin A depletion period. On Day -50, blood was collected for a baseline plasma vitamin A concentration. This sample was sent to Michigan State University (MSU Veterinary Diagnostic Laboratory, Lansing, MI) for evaluation using ultra high performance liquid chromatography (UPLC). The second sample on Day -35, was used for an in-house plasma biochemistry panel (VetScan VS1 Chemistry Analyzer, Abaxis, Inc., Union City, CA). Two separate sampling periods were required because of the blood volume required for UPLC and difficulties in drawing enough blood at one time. For the four replacement geckos, a baseline vitamin A plasma concentration from MSU was not performed and the samples for the biochemistry profile was obtained on Day -14. An additional blood sample was drawn during the course of the study on Day 35 for a repeat plasma biochemistry profile. On Days 105 and 140, samples from a single individual gecko were pooled together for a final plasma vitamin A concentration using UPLC from MSU.

4.2.4. Feeding experiments

The 24 geckos were randomly divided into three diet groups using a random number generator (random.org). The treatment groups received either vitamin A gut loaded BSF larvae that were intact (Group 1, n=8) or pierced once with a 21-gauge needle (Group 2, n=8). The control group (Group 3, n=8) received only non-gut loaded BSF larvae that remained intact. BSF larvae were gut loaded for 24 hours using a wheat

bran and corn meal based diet with an added water-soluble vitamin A supplement (Rovimix-A 500-WS [150,000 µg/g]; DSM Nutritional Products, Ames, IA). The expected final concentration of vitamin A in the diet was 20,000 µg/kg (dry matter basis) and the final larval vitamin A concentration was expected to be 1,000 µg/kg (as fed basis) (Boykin et al., 2018b). Over the course of the study, six samples of the wheat bran diet and larvae were sent to SDK Laboratories, Inc. (Hutchison, KS) for vitamin A concentration analysis performed by high performance liquid chromatography (HPLC). All geckos were offered larvae equal to 5% of their body weight three times per week. The amount ingested was recorded to determine how much vitamin A was ingested over the study period by the treatment groups.

4.2.5. Apparent digestibility of BSF larvae

In order to measure how well leopard geckos digest BSF larvae, all fecal samples produced during the course of the feeding trial (Days 0-140) were collected and pooled together by feeding group. Any water that contained feces was first placed in a glass dish and dried in an oven at 100°C for several hours before being combined with the rest of the fecal material. Fecal samples were frozen at -80°C until they could be further processed. Nutrient content analysis was performed by Dairy One Forage Lab (Ithaca, NY). Due to the small quantities of feces produced, only three pooled samples were collected for each diet group. Nutrient analysis was also performed on representative samples of BSF larvae (gut loaded and control). Average daily intake was determined for each group of geckos and apparent nutrient digestibility was calculated based on the dry matter intake and excretion of each nutrient using the following formula:

$$\text{Apparent digestibility (\%)} = \frac{\text{Average daily intake} - \text{Average daily output}}{\text{Average daily intake}} \times 100$$

4.2.6. Liver biopsies

At the conclusion of the study, all geckos underwent anesthesia and surgical liver biopsies for determination of liver vitamin A concentrations. Geckos were fasted for 12 hours prior to pre-medication with dexmedetomidine (0.1 mg/kg), midazolam (1 mg/kg), and hydromorphone (2 mg/ml, Hospira, Inc., Lake Forest, IL) at 0.25 mg/kg subcutaneously in the axillae. The geckos were maintained on isoflurane inhalant gas via face mask during the procedure. Anesthesia was monitored throughout the procedure via respiratory rate, Doppler heart rate, and muscle tone. The surgical site was prepared aseptically with chlorhexidine scrub 2% and sterile saline. A #11 scalpel blade was used to make a 1-1.5 cm left paramedian incision starting 1-1.5 cm caudal to the xiphoid process. Both lobes of the liver were visually assessed to look for any abnormalities. The left lobe was then gently exteriorized using sterile cotton tipped applicators and digital manipulation. The caudal half of the left lobe was clamped using Hemoclips (blue or medium size, Weck Hemoclip® Traditional; Teleflex Medical, Research Triangle Park, NC) and removed with a scalpel blade, placed in a Whirl-Pak® bag (Nasco, Fort Atkinson, WI), and frozen at -20°C until processed. The surgical site was closed using a 2-layer method and 4-0 Vicryl suture (polyglactin 910, Ethicon US, LLC, Somerville, NJ); the body wall was closed in a simple continuous pattern and the skin was closed using a horizontal mattress pattern.

The geckos were reversed with atipamezole following the same dose protocol noted previously. Meloxicam (OstiLox ^{TM}, VetOne, Boise, ID) at 0.2 mg/kg subcutaneously was also given post-operatively. A second dose of hydromorphone (0.25 mg/kg) was given 18-24 hours after surgery, and the meloxicam at 0.2 mg/kg

subcutaneously was continued once per day for four days. Seven geckos (Group 1, n=3; Group 2, n=2; Group 3, n=2) were euthanized intra-operatively after collection of the biopsies due to marked weight loss toward the end of the study period. All seven were submitted for full necropsy.

4.2.7. Statistical analysis

Sample size for this study was determined using the following a priori information: an alpha = 0.05, a power = 0.80, an expected difference in vitamin A liver concentrations of 20 µL/g, and a standard deviation for the treatment and control groups of 12 µL/g. The Shapiro-Wilk test, skewness, kurtosis, and q-q plots were used to evaluate the distributions of the data. Non-normal data were log transformed for parametric testing. Outliers were identified using the Dixon's Q test and removed if the calculated Q value was larger than the critical value given a 95% confidence interval. The amount of vitamin A ingested per group was dependent on voluntary gecko intake, therefore, this amount was analyzed for significance between groups using a one-way ANOVA. Fecal digestibility coefficients were also analyzed using a one-way ANOVA. Paired plasma biochemistry data was analyzed using a repeated measures ANOVA. For final vitamin A concentrations in the plasma and liver, Groups 1 and 2 were combined into a single vitamin A gut loaded group versus the non-gut loaded control group (Group 3). The two groups were then analyzed using a one-tailed independent t-test. A commercial software (SPSS 25.0; IBM Statistics, Armonk, NY) was used to analyze the data. A $p<0.05$ was used to determine statistical significance.

4.3. Results

4.3.1. Physical exams and necropsy results

All geckos were determined to be healthy at the beginning of the study. Over the course of the experiment, 10 (41.6%) out of 24 leopard geckos experienced an overall loss from their starting weights (mean ± SD: -13.76 ± 7.71%, range: -0.14% to -23.91%). Seven (29.2%) of these geckos experienced inappetence and weight loss severe enough to require early removal from the study. These geckos were euthanized intra-operatively (after collection of hepatic biopsies) and then submitted for necropsy. Necropsy results revealed stomatitis (n=4 total; mild to moderate, n=3; moderate to marked, n=1), hepatic lipidosis/vacuoles (n=4), infection with *Cryptosporidium* spp. via histopathology and/or fecal floatation (n=3), lymphoplasmacytic enteritis (n=3), renal tubular necrosis (n=2), and pulmonary xanthomatosis (n=1). None of the necropsied specimens had evidence of epithelial squamous metaplasia that could be associated with hypovitaminosis A.

4.3.2. Ophthalmic exams

No obvious ophthalmic lesions were observed at the beginning of the study. Exams were repeated at the end of the study. Seven (29.2%) of the geckos did have mild mucoid discharge within the medial canthus of one or both eyes. Three of these animals were from the necropsied group and showed no evidence of squamous metaplasia or any other ocular disease. Correlation analysis was done and showed no significant correlation between the presence of the discharge and the liver or plasma vitamin A concentrations. The authors are unsure as to the significance of the mucoid discharge, as no other significant ocular disease process was found.

4.3.3. Diet vitamin A concentrations and intake amounts

The target vitamin A concentrations for the larval diet and larvae were 20,000 µg/kg (dry matter basis [DMB]) and 1,000 µg/kg (as fed), respectively. When analyzed samples were averaged together over the course of the experiment, the larval diet was 32,753 ± 15,350 µg/kg (DMB) and the larvae for the two treatment groups were 835 ± 232 µg/kg (as fed). The average vitamin A concentration of the control group diet and larvae were 391 ± 40 µg/kg (DMB) and 23 ± 30 µg/kg (as fed), respectively. Geckos were allowed to eat the larvae *ad libitum* up to 5% of their body weight. On average, the geckos ate 54.7 ± 12.2% of the food offered to them per feeding (2.74% body weight per feeding). When intake was analyzed as the average amount of grams eaten per group per feeding, there was no significant difference ($F=0.587$, $p=0.558$).

4.3.4. Apparent digestibility of BSF larvae

The mean digestibility coefficient ± standard deviation for each group is listed in Table 4.1. There was no significant difference between groups in regards to digestibility for any of the nutrients analyzed.

4.3.5. Bloodwork values

Baseline plasma vitamin A samples were all found to be <50 ng/ml (lower limit of quantification for small volume samples, <1 ml). For the final plasma vitamin A samples only 13 out of 24 samples were of a high enough volume to give readings above the limit of quantification. Vitamin A was significantly higher ($t=1.906$, $p=0.0415$) in the treated groups (n=8; 33.38 ± 7.11 ng/ml) compared with the control group (n=5; 25.8 ± 6.72 ng/ml) (Table 4.2).

Table 4.1. Apparent digestibility coefficients for leopard gecko fed an exclusive diet of BSF larvae prepared in one of three ways.

Nutrient	Group 1: Intact, vitamin A gut loaded	Group 2: Pierced, vitamin A gut loaded	Group 3: Intact, non-gut loaded	Significance between groups
Dry Matter, %	70 ± 0.88	72 ± 9.3	71 ± 4.5	F=0.061, p=0.942
Crude Protein, %	81 ± 0.48	82 ± 3.8	80 ± 1.7	F=0.178, p=0.845
Crude Fat, %	65 ± 11.7	74 ± 13.7	67 ± 5.9	F=0.361, p=0.724
Ash, %	49 ± 9.3	54 ± 11.3	54 ± 6.1	F=0.203, p=0.827
Calcium, %	41 ± 8.9	42 ± 18.1	44 ± 6.1	F=0.029, p=0.972
Phosphorous, %	42 ± 4.1	47 ± 10.8	46 ± 3.2	F=0.255, p=0.790
Magnesium, %	50 ± 5.8	52 ± 8.8	52 ± 3.0	F=0.088, p=0.918
Potassium, %	77 ± 1.4	81 ± 2.9	80 ± 3.4	F=1.081, p=0.443
Sodium, %	62 ± 3.9	65 ± 13.8	17 ± 90.9	F=0.520, p=0.640
Iron, mg/kg	58 ± 4.9	60 ± 2.6	58 ± 2.8	F=0.105, p=0.903
Zinc, mg/kg	37 ± 5.4	39 ± 1.0	43 ± 2.5	F=1.402, p=0.372
Copper, mg/kg	26 ± 6.0	31 ± 4.4	33 ± 0.3	F=1.323, p=0.387
Manganese, mg/kg	24 ± 8.1	20 ± 15.9	27 ± 5.1	F=0.214, p=0.819
Molybdenum, mg/kg	44 ± 8.5	49 ± 10.9	52 ± 2.1	F=0.502, p=0.648
Sulfur, mg/kg	41 ± 0.7	47 ± 9.0	45 ± 4.5	F=0.482, p=0.659

Table 4.2. Plasma and liver vitamin A concentrations from geckos receiving vitamin A gut loaded BSF larvae versus those receiving non-gut loaded BSF larvae.
All samples were run by Michigan State University's Veterinary Diagnostic Laboratory using UPLC. Eleven plasma samples were not included due to insufficient plasma quantities reading below the limit of quantification for this assay (LOQ: <50 ng/ml).

	Vitamin A supplemented (Groups 1 & 2)	Non-gut Loaded (Group 3)
Final Plasma Vitamin A concentrations (ng/ml)	33.38 ± 7.11 (n=8)	25.80 ± 6.72* (n=5)
Final Liver Vitamin A concentrations (µg/g)	28.67 ± 18.90 (n=16)	14.13 ± 7.41** (n=7)

*One sample was reported as <20 ng/ml. This value was included as a reading equal to 20 ng/ml.
**A single outlier of 61.35 µg/g was removed from this group prior to statistical analysis.

Plasma biochemistries were sampled on Day -35 (baseline) and Day 35. A final sample was not performed due to the plasma volume restrictions of the vitamin A test. No significant differences were seen between groups during either of the two time periods; however, when baseline values (averaged across all three groups) were compared to Day 35 values using a repeated measures ANOVA, there were significant decreases in calcium, total protein, albumin, globulin, and sodium (see Table 4.3).

Table 4.3. Biochemistry results from leopard geckos at baseline and on Day 35. Mean ± standard deviation is reported, n=24. F statistics and p values are reported for analytes that showed significance.

Biochemistry Analytes	**Baseline Values**	**Day 35 Values**	**Significance**
AST (U/L)	54.6 ± 20.3	67.91 ± 37.17	
Bile acids (Umol/L)	<35 ± 0	<35.4 ± 1.88	
Creatinine kinase (U/L)	693.3 ± 669.8	1150.43 ± 1847.6	
Uric acid (mg/dL)	3.37 ± 1.45	3.8 ± 1.40	
Glucose (mg/dL)	166.5 ± 14.36	161.61 ± 20.12	
Calcium (mg/dL)	14.16 ± 1.1	12.98 ± 1.05	F=25.299, p<0.001
Phosphorous (mg/dL)	3.82 ± 0.63	3.64 ± 0.75	
Total Protein (g/dL)	5.85 ± 0.88	5.11 ± 0.74	F=19.061, p<0.001
Albumin (g/dL)	1.78 ± 0.31	1.46 ± 0.28	F=30.076, p<0.001
Globulin (g/dL)	4.07 ± 0.68	3.66 ± 0.5	F=10.717, p=0.004
Potassium (mmol/L)	5.22 ± 0.60	4.96 ± 0.79	
Sodium (mmol/L)	147.8 ± 5.75	138.8 ± 3.1	F=94.955, p<0.001

4.3.6. Liver biopsies and surgical outcomes

Liver biopsies collected at the end of the study revealed a significantly higher vitamin A concentration for the gut loaded group versus the control group (Vitamin A supplemented: 28.67 ± 18.90 µg/g, Non-gut loaded: 14.13 ± 7.41 µg/g; t=1.951,

p=0.0325) (see Table 4.2). A single outlier was removed from the control group (liver vitamin A concentration of 61.35 µg/g). Age, genetic factors, or diet prior to the study could have played a role in the unusually high liver concentration of this gecko compared to the rest of the geckos in this group. There were no anesthetic complications with any of the surgeries. All non-survival surgeries (n=7) were determined prior to the start of anesthesia. All survival surgeries (n=17) were performed successfully with only one animal requiring the placement of Gelfoam® Sterile Sponge (Pfizer Inc., New York, NY) for more controlled hemostasis. Post-operatively, there were no issues with hemorrhage, dehiscence, or any other wound complications. As of twelve weeks post-surgery, no additional issues or concerns were noted.

4.4. Discussion

The results of this study confirm that leopard geckos are capable of digesting BSF larvae. Overall, BSF digestibility was higher in this reptile species compared to a single study with an amphibian (Dierenfeld and King, 2008). Average dry matter digestibility of whole BSF larvae was 71 ± 2.8% when fed to leopard geckos and 26 ± 9.9% for mountain chicken frogs. Significant gains in digestibility were also seen for protein, magnesium, potassium, sodium, iron, zinc, copper, and molybdenum. These differences were likely due to a higher degree of mastication by the leopard geckos, allowing for digestive enzymes to breach the tough exoskeleton and breakdown the inner portions of the larvae. This would also explain why piercing the BSF larvae with a needle did not result in improved digestibility between the three groups, as the geckos were already accomplishing this through mastication. It is possible that needle-piercing would still be of benefit to species that swallow their prey whole. Transecting the BSF larvae could

also be considered, but this led to decreased movement by the larvae and reduced acceptance rate by the geckos. Calcium digestibility, on the other hand, did not improve and was similar between the two species (43% for geckos, 44% for frogs). It would appear that mild levels of mastication or needle piercing is not sufficient to release the calcium carbonate bound in the exoskeleton matrix. Visual assessment of the geckos' feces supports this hypothesis, as there would frequently be whole exoskeletons passed through the digestive tract. This poor level of calcium digestibility should raise concerns over whether non-supplemented BSF larvae can support the calcium needs of captive reptiles.

When comparing the paired blood samples, there was a significant decrease in calcium over time. Poor calcium digestibility is a possible cause for this decline. Another explanation could be that the geckos received high levels of calcium supplementation prior to entering the study and that the decline represented a return to more physiologic levels. Unfortunately, due to plasma volume restrictions at the conclusion of the study, a final biochemistry panel to determine whether or not values continued to trend down could not be performed. As all of the geckos were males, reproductive status did not play a role in this decline. Additional research needs to be done to establish true calcium requirements for reptiles, but based on these results, the authors recommend that BSF larvae receive calcium dusting or gut loading prior to being fed to any captive reptile.

The biochemistry panels also revealed significant decreases in total protein, albumin, globulins, and sodium in the geckos over time. Compared to other feeder insects, BSF larvae have lower concentrations of protein and sodium (Finke, 2013). The

drop in protein could indicate a change from a primarily protein-rich cricket diet to one of a higher fat, lower protein larval diet. Hyporexia could also play a role in the decreased values, but with an average intake of 2.7% body weight and no correlation between blood values and the amount of food ingested, this is less likely. In regards to sodium, there is added concern about the possibility of poor or negative digestibility. Negative digestibility indicates that more sodium is being lost in the feces than was initially present in the food. This was seen in both mountain chicken frogs and corn snakes (*Pantherophis guttatus*) fed diets containing BSF larvae (Dierenfeld and King, 2008; Boykin et al., 2018a). It was also seen in a single replicate from Group 3 of this study. Dierenfeld and King originally theorized that the BSF larvae may be irritating the gut and causing a "diarrhea"-type syndrome that could lead to hyponatremia if fed exclusively over time. Unfortunately, no other studies have addressed digestibility of sodium or looked at sodium on biochemistry data from animals ingesting BSF larvae. To the authors' knowledge, the present study is the first to report sodium concentrations for an animal consuming either whole BSF larvae or as the main ingredient of the diet. When compared to reference intervals for this species, the sodium levels obtained from Day 35 of this study do indicate a possible hyponatremia (Alberton et al., 2018; Mitchell-unpublished data), however, these reference intervals may not be representative of the true physiologic range for sodium in leopard geckos. Feeding a variety of insects or protein sources, should alleviate any concerns that could be associated with the nutritional deficits of an exclusive BSF larval diet.

Proving the second hypothesis regarding digestion and absorption of vitamin A from gut loaded larvae was a little more challenging to confirm. Previous research in

multiple animal species suggested that plasma vitamin A concentrations are not usually representative of whole body vitamin A status, nor do they correlate well with liver vitamin A concentrations (Berkvens et al., 2014; Dutton et al., 2014; Sullivan et al., 2014). Retinol-binding proteins within the blood tend to maintain homeostatic concentrations of circulating vitamin A, except in cases of extreme hypo- or hypervitaminosis A (D'Ambrosio et al., 2011; Schweigert et al. 1991). Liver concentrations are considered the more accurate representation of body status, but clinically can be more challenging to obtain in sick or small patients. Ideally, liver biopsies from each gecko would have been obtained at the beginning and end of the study period. Without knowing starting concentrations of liver vitamin A for each gecko, it is impossible to truly know whether the treatment groups experienced a change in vitamin A concentration compared to the control group; however, the random assignment of the geckos to their respective groups and the defined dietary assignments were done to control for this limitation. Unfortunately, due to the geckos' small size and the minimum requirements for biopsy weight (0.25 grams per MSU), only one hepatic biopsy would be able to be performed unless the study became non-survival. The other issue was the possibility of fatal complications early on in the study period leading to reduction in sample size populations. A recent study reported a high complication rate following liver biopsies in this species, so the authors had concerns about multiple surgeries (Cojean et al., 2018). Due to these limitations and the fact that there are no published references for plasma vitamin A in leopard geckos, the authors decided to proceed with collecting plasma concentrations as well.

The minimum plasma volume required for the vitamin A assay used in this study was 0.15 ml; however, the limit of quantification for sample volumes below 1 ml is 50 ng/ml. For samples that are larger than 1ml, the limit of quantification can go as low as 10 ng/ml. Unfortunately, due to size restrictions, our baseline sample volumes all fell below 1 ml of plasma and all results were returned as <50 ng/ml. Previous literature has reported plasma vitamin A concentrations for various reptile species, including green iguanas (*Iguana iguana*) (52-75 ng/ml), eastern indigo snakes (*Drymarchon couperi*) (9 ng/ml), anacondas (*Eunectes murinus*) (80 ng/ml), alligator snapping turtles (*Macrochelys temminckii*) (192 ng/ml), eastern box turtles (*Terrapene carolina*)(221 ng/ml), pancake tortoises (*Malacochersus tornieri*) (450 ng/ml), and leatherback sea turtles (*Dermochelys coriacea*)(500 ng/ml) (Calle et al., 1994; Chaffin et al., 2008; Deem et al., 2006; Holladay et al., 2001; Knafo et al., 2016; Raila et al., 2002; Raphael et al., 1994). To the authors' knowledge, there is no other literature reporting plasma vitamin A concentrations in leopard geckos, or any other insectivorous lizard, so these baseline values either indicate that leopard geckos have lower than average circulating plasma vitamin A concentrations compared to other reptiles, including other squamates, or that these geckos were already deficient prior to being included in this study. Given that all individuals were deemed healthy at the beginning of the study, and that no geckos developed lesions that would be considered classical for diagnosis of hypovitaminosis A (epithelial squamous metaplasia), the former is probably more likely.

Only 13 of the 24 final plasma samples were reported back with values reading below 50 ng/ml (Group 1, n=7; Group 2, n=1; Group 3, n=5). Thus, for comparative analysis between treatments, data from Groups 1 and 2 were combined, making for a

single vitamin A gut loaded group versus the control group (Group 3). Final plasma concentrations did show a significant difference between groups ($t=1.906$, $p=0.0415$), with the vitamin A supplemented geckos having higher plasma vitamin A concentrations. Liver vitamin A concentrations were also significant between the vitamin A gut loaded groups and the control group ($t=1.951$, $p=0.0325$). As mentioned previously, a single outlier was removed from the control group for having an abnormally high vitamin A concentration. One individual in the gut loaded group also had an abnormally high vitamin A concentration, but did not meet the requirements for removal using the Dixon's Q test with a 95% confidence interval. However, significance would have remained with that value removed as well. These individuals likely had higher liver concentrations of vitamin A at the start of the study compared to the other individuals. Age of the geckos and husbandry prior to their inclusion in the study are unknown. These individuals may have received diets much higher in vitamin A (silkworms, different brand of gut loading or dusting supplements, etc.) when being managed as breeders. Paired liver samples would have helped to identify these potential outliers at the beginning of the study, but due to the limitations already discussed, were not performed.

One other study has measured liver vitamin A concentrations in leopard geckos. Cojean et al. (2018) used female leopard geckos to determine if differences existed between liver vitamin A uptake and storage when geckos were given pre-formed vitamin A supplementation versus beta-carotene. Similar to the present study, Cojean's group also only performed biopsies at the end of the study and did not collect paired samples. Those authors found that the beta-carotene group had higher overall liver concentrations of vitamin A compared with the vitamin A group (mean 13.43 µg/g, range 2.31-24.05

µg/g vs. mean 9.49 µg/g, range 6.76-13.33 µg/g, respectively), despite the theory that many carnivorous/omnivorous reptiles are not capable of converting beta-carotene to the active form of vitamin A. The values from the present study are much higher (vitamin A group mean= 28.67 ± 18.9 µg/g, range 2.9-77.98 µg/g; control group mean= 14.13 ± 7.4 µg/g, range 5.93-28.03, outlier 61.35 µg/g). The difference in sexes between the studies may be a major factor for these differences. Cojean et al. reported folliculogenesis as a complication to their study. Females would potentially have lower body stores of vitamin A due to large quantities being stored in the developing eggs. Additionally, age was likely a contributing factor as well. A majority of the geckos used by Cojean's group were 6-9 months of age. The male geckos used in the current study were thought to have been mostly adults (>10 months) based on size. To a certain extent, vitamin A tends to accumulate in the liver as an animal ages, leading to potential differences in the two populations (van der Loo et al., 2004). In contrast to their study, we experienced no major surgical or anesthetic complications. Seventeen geckos underwent a successful liver biopsy of the left lobe (median weight= 0.22g, min= 0.08g, max=0.59g). Lower overall vitamin A stores or some other aspect of folliculogenesis may have contributed to poor wound healing and dehiscence. Additionally, different anesthetic protocols and biopsy techniques (guillotine vs. Hemoclip) may have led to better success in the present study. The authors would strongly encourage further studies involving liver biopsies on leopard geckos in the future, if they are indicated. One such study would be to determine plasma and liver vitamin A concentrations in wild-caught leopard geckos. This much needed study would help researchers to determine if our various methods of supplementation in captivity are meeting the physiological needs of this species. As far

as which supplementation (beta-carotene or vitamin A) is better for leopard geckos, there is still some debate, but both supplements appear to have some degree of absorbance and assimilation into the gecko and should probably be used in combination.

Without supplementation with either vitamin A or beta-carotene, reptiles will eventually become at risk for developing hypovitaminosis A. The lesions most often associated with this disease are hyperkeratosis and epithelial squamous metaplasia, with the most classical presentations manifesting as ocular lesions (periocular edema, conjunctivitis, blepharitis, ocular discharge and debris) (Abate et al., 2003; Boyer, 2019; Mans and Braun, 2014). Wiggans et al. (2018) reported that 46% of all leopard geckos that visited a veterinary teaching hospital between 1985 and 2013 had some form of ocular lesion. Lack of vitamin A in the diet was one of the major risk factors associated with development of ocular disease. In the present study, mild mucoid discharge was seen in seven geckos. However, the individual geckos were spread across all three groups and there was no correlation between presence of discharge and either plasma or liver vitamin A concentrations.

Other symptoms may vary by species and have included nasal discharge, gular edema, swollen or thickened lips, stomatitis, vertebral kinking, hemipenal impaction, and renal tubular hyperkeratosis leading to visceral gout (Abate et al., 2003; Ariel et al., 1997; Ferguson et al., 1996; Mans and Braun, 2014). The timeline for developing these lesions is still currently unknown. Kroenlein et al. (2008) found no difference in liver vitamin A concentrations nor histological evidence of squamous metaplasia in red-eared sliders (*Trachemys scripta elegans*) after six months of vitamin A depletion. Research in humans and other adult vertebrates confirm that >6 months of depletion is needed before

clinical signs of hypovitaminosis A are detectable (Hume and Krebs, 1949; McCarthy and Cereoedo, 1952). None of the seven geckos (Group 3, n=2) that underwent necropsy had histopathological lesions consistent with hypovitaminosis A. A case could be made for stomatitis lesions being associated with hypovitaminosis A, but lesions were seen in treatment and control groups and were not correlated to vitamin A concentrations. Even though there was a significant difference seen between groups in regards to liver and plasma vitamin A concentrations, without histopathological evidence of disease, the authors are unable to conclude that the control group was or was trending towards vitamin A depletion status. The difference in concentrations only provides evidence that gut loaded BSF larvae are capable of being digested enough to provide vitamin A to the consumer and to produce levels higher than geckos not receiving any supplementation.

Although BSF larvae appear to be well digested and able to offer gut loaded nutrients to leopard geckos, 10 out of the 24 individuals developed issues with inappetence and weight loss during the course of the study. The reason for this was likely multi-factorial. One of the issues was food preference on the part of the leopard geckos. Even before the study started, four geckos had to be replaced due to strict refusal to eat BSF larvae. Most of the geckos that experienced weight loss during the study period would consistently eat less than the other geckos. Whether it was due to palatability or a lack of movement compared to crickets, could not be answered in this study, but this food preference was a major contributor to the weight loss seen. Another contributor was infection with *Cryptosporidium* sp. At least three geckos were confirmed to be infected by histopathology and fecal floatation at time of necropsy (Group 1, n=2; Group 3, n=1). The geckos that were infected did not start showing obvious signs of

infection until after the acclimation period had been completed. The stress of being transitioned to an exclusive diet of BSF larvae, instead of having intermittent feedings with crickets, may have increased the severity and progression of the disease. Knowing that the prevalence of cryptosporidiosis is relatively high in the commercially bred population of leopard geckos, the authors were well aware that this parasitic disease could disrupt results for a digestibility study. Fecal floatations performed at the beginning of the study did not reveal the infection. Financial limitations of the study kept us from being able to run a PCR for each individual gecko or from obtaining geckos that could be classified as pathogen-free; these animals ultimately represent what is available in the current pet trade. Fortunately, the geckos that were infected rarely ate well and, therefore, did not contribute much to the pooled feces. Overall, the results indicated good digestibility with the few exceptions noted earlier, and likely didn't impact the final conclusions. The other possible contributor that was found on necropsy was stomatitis. These lesions were not seen grossly, but inflammation was seen on histopathology. The cause of the stomatitis is unknown, but nutrient deficiencies or irritation from ingestion of the BSF larvae are possible. Once again, the authors strongly encourage feeding insectivores a wide variety of insects in order to provide the diverse nutrients they require.

4.4.1. Conclusions

Overall, leopard geckos are able to digest BSF larvae much better than previously was reported for the mountain chicken frog. Despite improvements with other nutrients, calcium digestibility was still poor, suggesting that the calcium bound within the exoskeleton is not easily broken down with simple mastication. Dusting or gut loading

these insects with calcium and multivitamin supplements is still recommended. This study demonstrated that leopard geckos are able to utilize gut loaded nutrients such as vitamin A from BSF larvae. Additional research is necessary to determine gut loading amounts for other nutrients (e.g., calcium, vitamins D_3 and E, among others). Plasma and liver vitamin A concentrations for leopard geckos can be challenging to obtain in a clinical setting, but can be used in research to better study true requirements for this species.

CHAPTER 5.
DEVELOPMENT OF A NOVEL INSECT-BASED SAUSAGE DIET FOR SNAKES

5.1. Introduction

Numerous species of snakes and other carnivorous reptiles are kept in captivity by both reptile hobbyists and zoological institutions across the globe. The species that are kept in captivity represent a large variety of different taxa, as well as diverse life strategies (Mitchell, 2009). These species can be highly variable in their dietary preferences, with some species commonly ingesting mice and other small mammals, while others prefer to eat fish, birds, amphibians, other reptiles, insects, eggs, or much larger prey species (Donoghue, 2006; Funk, 2006). Even within a specific taxonomic group, natural diets would consist of a variety of different prey items (Mitchell, 2009). Yet, many of the snake species kept in captivity are fed an almost exclusive diet of small mammals, such as mice and rats, due to the wide availability of these prey species from vendors and ease of feeding (Donoghue, 2006). However, these food items are not without their negative effects, as these prey species are known to injure snakes when offered as live prey and the high nutritional content of these prey species can lead to obesity in captive snakes (Mitchell, 2009). Efforts should be taken to increase the variety of diets offered to captive snakes to minimize the likelihood for injuries, control the number of calories being offered, and to expand the market of available diets for carnivorous reptiles.

Processing whole vertebrate prey into sausage casings with various scents has been performed by herpetoculturists over the years in an attempt to reduce feeding costs and increase the variety of protein sources being offered. However, to the authors'

knowledge, no study has yet been conducted to evaluate any health or growth parameters of snakes being offered an alternative diet, nor addressed the palatability or willingness of snakes to ingest such a diet. The willingness of snakes to accept this form of diet would open the market for food companies to start producing commercially developed food options for snakes. Commercially-produced diets, as compared to various whole prey items, would offer the advantages of increased variety being widely available to consumers, as well as the ability to alter the diet's components to meet the desired nutritional needs of a snake. Once alternative diets have proven themselves to be both safe and well-accepted through feeding trials, more specific diets can be developed to meet various needs such as leaner meats to aid in weight loss of obese snakes, low purine diets to assist in the treatment of gout, or supplementation of antioxidants or specific vitamins to help reptiles combating illnesses such as liver disease or thiamine deficiency (De Voe, 2014; Donoghue, 2006; Mitchell, 2009).

Development of non-traditional sources of protein, such as insects that can be raised on feed by-products and other waste materials, allows for more efficient use of limited resources and less dependence on vertebrate protein sources, as well as allowing for a greater diet variety for our captive reptiles (Barragan-Fonseca et al., 2017; Oonincx et al., 2015). Additionally, a non-vertebrate protein source has the advantage of appealing to reptile owners that are not comfortable feeding mice and other vertebrate prey to their animals. Black soldier fly (BSF) larvae have already been studied and used as ingredients in feeds for poultry, swine, alligators, and aquaculture due to their high protein value and feed conversion efficiency (Barragan-Fonseca et al., 2017). They are also gaining popularity as a feeder insect for insectivorous reptiles due to their naturally

high calcium to phosphorous ratio (Barragan-Fonseca et al., 2017; Dierenfeld and King, 2008). In addition to their beneficial nutrient composition, they are also known to be pathogen-free and have little to no risk of harboring harmful bacteria such as *E. coli* or *Salmonella* spp. (Erickson et al., 2004). At least two major recalls of frozen feeder mice have occurred in the United States over the last ten years due to human illnesses associated with *Salmonella* Typhimurium (Harker et al., 2011; Lee et al., 2008). By using insects as a main protein source, we have the potential to reduce this ongoing zoonotic threat.

The overall goal of this project was to determine if snakes fed a commercially-produced diet would maintain similar growth and health parameters as seen in snakes being fed a more traditional diet. We hypothesized that there would be no significant differences in growth, physical examination findings, or nutrient digestibility in snakes offered an insect-based diet versus a standard mouse diet, and that with proper scenting and a short acclimation period, that there would be no significant differences in palatability between the two diets.

5.2. Materials and Methods

5.2.1. Animals and housing

Nine sub-adult male corn snakes (*Pantherophis guttatus*) (2 months of age, 12.9-16.2 grams) were obtained from a commercial breeder and acclimated to laboratory conditions over a period of four weeks. Male snakes were selected to eliminate the effect of sex on the results because of the limited sample size in this pilot study. Each snake was housed in an individual plastic enclosure (43 cm x 30 cm x 25 cm) and provided a 12

hour photoperiod. The ambient temperature and humidity ranges were 28.3-30° C (83-86° F) and 30-40%, respectively. All snakes had free access to water at all times.

5.2.2. Diet

Using the nutritional compositions of vertebrate protein sources as a guide, a commercially-produced sausage diet was formulated using BSF larvae as a novel protein source (Dierenfeld et al., 2002) (see Table 5.1). The nine snakes were randomly divided (random number generator; random.org) into two feeding groups for this cross-over study. Group 1 was offered frozen-thawed mice over a two-month period and then switched to the insect-based sausage diet for the following two months, for a total of a four month feeding trial period. Group 2 received the sausage diet over the first two months and then switched to mice over the last two months of the study. The snakes were weighed twice weekly just prior to feeding and were offered food items that were equal to 15% of their current body weight. Each meal was offered twice, 24 hours apart, before it was considered to be a failed feeding. For each feeding attempt, up to three different scent options were applied in sequence to try and entice a feeding response. Scent options that were attempted over the course of the experiment included plain/no scent added, freeze dried mouse powder, mouse blood, mouse brain, and pinkie skins. For those snakes that skipped two meals in a row, the diet being offered was force-fed using a pinkie pump (LLLReptile and Supply Co., Inc., Oceanside, CA) and an 18-French red rubber catheter (Becton Dickinson, East Rutherford, NJ).

Palatability was assessed by recording the number of meals eaten willingly, as well as measuring the time taken to strike the food once presented (up to 5 minutes).

Table 5.1. Nutritional content of the experimental sausage diet versus common whole vertebrate prey fed to juvenile snakes. Values presented in this table represent a combination of data obtained from the current experiment and from previous literature (Dierenfeld, 2002). All values are expressed as mean ± standard deviation unless only one value was available.

	BSF larvae sausage	**Neonatal mouse (<3g)**	**Juvenile mouse (3-10 g)**	**Domestic chicken (one day old chick)**	**Anole lizard**
DM %	42.5 ± 0.93	20.9 ± 0.61	24.1 ± 4.49	24.2 ± 1.98	28.3 ± 1.56
Crude protein %	45.9 ± 4.85	63.1 ± 0.30	50.4 ± 6.57	66.3 ± 1.98	66.7 ± 0.99
Crude fat %	23.3 ± 0.86	28.5 ± 1.00	32.0 ± 6.40	19.5 ± 4.17	9.00
Ca %	1.77 ± 0.09	1.51 ± 0.04	1.95 ± 0.74	1.71 ± 0.03	3.92 ± 2.29
P %	1.21 ± 0.10	1.79 ± 0.03	1.88 ± 0.23	1.22 ± 0.01	2.74 ± 0.20
Ca/P ratio	1.46 ± 0.12	0.85 ± 0.02	1.12 ± 0.43	1.41 ± 0.03	1.40 ± 0.73
Mg %	0.19 ± 0	0.1 ± 0.01	0.10 ± 0.02	0.07 ± 0.02	0.15 ± 0.01
Na %	0.28 ± 0.01	0.60 ± 0.02	0.46 ± 0.03	0.77 ± 0.08	0.42 ± 0.12
K %	1.24 ± 0.03	1.20 ± 0.02	1.00 ± 0.03	0.81 ± 0.01	0.87 ± 0.19
Cu, mg/kg	22.5 ± 11.6	12.7 ± 0.58	13.1 ± 1.40	4.6 ± 0.85	179 ± 246
Fe, mg/kg	356 ± 22.4	170 ± 7.00	222 ± 82.8	138 ± 26.8	131 ± 4.38
Zn, mg/kg	65.8 ± 1.71	94.0 ± 2.00	81.0 ± 10.4	95.7 ± 2.47	229 ± 122
Gross energy kcal/g	4.42 ± 0.08	4.85 ± 0.07	6.25 ± 0.57	5.81 ± 0.01	4.80

Additional measurements that were collected included the frequency of regurgitation events following force feeding and the amount of food regurgitated.

5.2.3. Physical examinations and growth measurements

A baseline physical examination was performed on each animal at the beginning of the study. Initial body weight, body condition score (BCS), snout to vent length (SVL), tail length (TL), and body girth at the neck and at ¾ of the SVL were determined. These same measurements were collected again at the two month cross over point and at the conclusion of the study.

5.2.4. Nutrient digestibility

All fecal samples over the four-month period were collected and pooled together by diet group and time period. Any water that contained feces was first placed in a glass dish and dried in an oven at 100°C for several hours before being combined with the rest of the fecal material. Fecal samples were then analyzed for nutrient content by Dairy One Forage Lab (Ithaca, NY). Due to the small quantities of feces produced, only one sample was collected from each diet group during the first two-month period. During the second half of the experiment, two samples from each diet group were collected and the data averaged. Nutrient analysis was also performed on representative samples of the insect sausage and frozen mouse diets (sausage diet, n=6; pinkie mice, n=10; peach fuzzy mice, n=7; fuzzy mice, n=4; and pinkie skins, n=32). Values were averaged together for each food category and the average daily intake for each of the categories was determined. Apparent nutrient digestibility was calculated based on the dry matter intake and excretion of each nutrient using the following formula:

$$\text{Apparent digestibility (\%)} = \frac{\text{Average daily intake} - \text{Average daily output}}{\text{Average daily intake}} \times 100$$

5.2.5. Statistical analysis

The Shapiro-Wilk test, skewness, kurtosis, and q-q plots were used to evaluate the distributions of the data. Non-normal data were log transformed for parametric testing. Mixed linear models were used to determine if the growth data was affected by diet or time. Snake was entered into the model as the random factor, while diet type and sampling time were fixed factors. For digestibility data, statistical comparisons were not performed due to the limited amount and number of samples. Thus, descriptive statistics were used to present data for digestibility, as well as for presenting data on force feeding and regurgitation events. Regarding palatability data, the willingness to eat the sausage diet consistently (5 or more meals over the two-month period) versus the mouse diet was calculated across both groups of snakes using a Fischer's exact test. A commercial software (SPSS 25.0; IBM Statistics, Armonk, NY) was used to analyze the data. A $p<0.05$ was used to determine statistical significance.

5.3. Results

Physical examinations across all time periods revealed no abnormalities in any of the snakes. One of the snakes in Group 2 did die during the course of the sausage feeding portion of the study, but the cause of death was determined by necropsy to be associated with asphyxiation following force feeding and incomplete regurgitation of food material.

There was a significant difference in body weight over time ($F=14.083$, $p=0.003$), but not by group ($F=0.001$, $p=0.993$) (Figure 5.1). There were no significant differences in any of the other morphometrics by time or group (SVL, group: $F=0.008$, $p=0.930$, time: $F=2.465$, $p=0.167$; tail length, group: $F=0.978$, $p=0.361$, time: $F=0.604$, $p=0.467$;

neck girth, group: F=0.001, p=0.974, time: F=0.019, p=0.896; body girth, group: F=3.47, p=0.109, time: F=1.01, p=0.352).

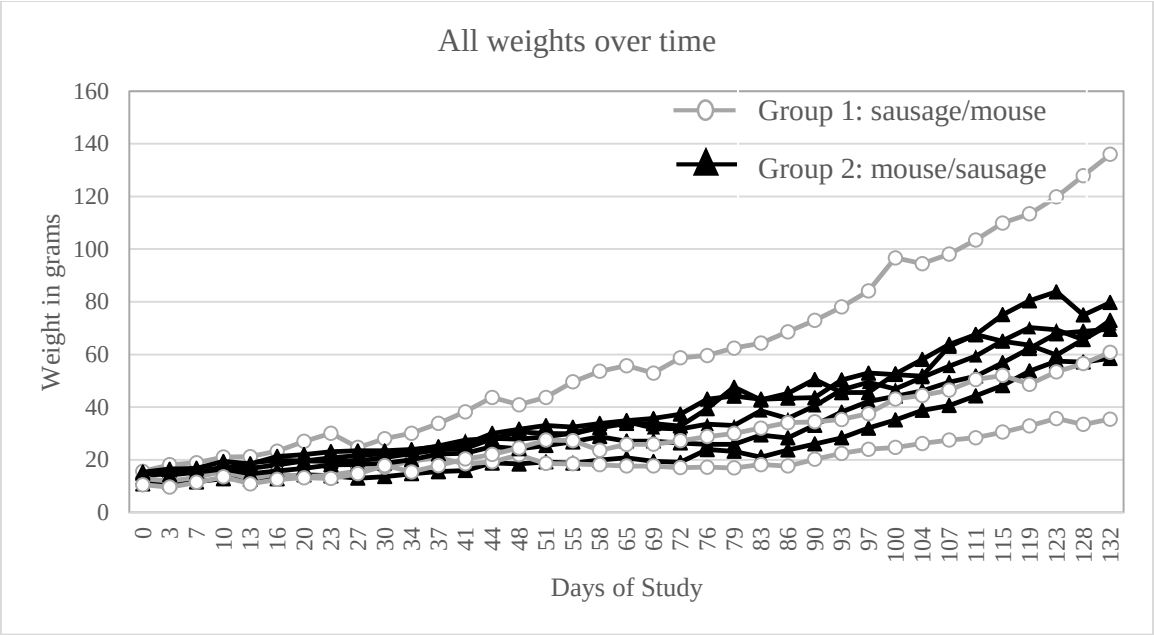

Figure 5.1. Weight in grams for all surviving corn snakes over the four-month time period. Group 1 snakes (n=3) were fed the sausage diet first. Group 2 snakes (n=5) were fed the frozen thawed mouse diet first. Days 65 represents the cross-over point. Significant differences in weight were not seen between groups for either time period, however, a significant difference was seen for all snakes over time.

The digestibility results are presented in Table 5.2. with a breakdown between each diet and time period. Most nutrients were greater than 50% digestible, suggesting that the snakes were able to absorb and utilize the nutrients from both diets. The nutrients that were less than 50% digestible in the sausage diet included sodium, magnesium, iron, and molybdenum, with sodium producing a negative apparent digestibility coefficient. The nutrients that were less digestible in the mouse diet were copper, manganese, and molybdenum, with manganese producing a negative apparent digestibility coefficient.

Table 5.2. Apparent digestibility coefficients for corn snakes fed either a frozen-thawed mouse diet or an insect-based sausage diet over two experimental periods.

	Part 1: mouse diet	Part 1: sausage diet	Part 2: mouse diet	Part 2: sausage diet
Dry matter, %	64.9	44.7	51.9	57.0
Crude protein, %	91.4	76.5	84.3	78.9
Crude fat, %	96.9	94.1	95.8	95.2
Ash, %	60.2	54.4	53.0	52.6
Ca, %	85.1	63.2	62.5	71.3
P, %	82.0	65.2	66.0	64.5
Mg, %	74.4	41.8	62.4	46.5
Na, %	100	- 54.7	30.3	- 49.6
K, %	81.2	71.7	77.0	65.0
Cu, mg/kg	7.52	55.1	33.5	62.8
Fe, mg/kg	47.1	0.00	56.2	21.0
Zn, mg/kg	82.8	66.2	77.0	59.4
Mn, mg/kg	35.7	49.3	- 55.9	62.5
Mo, mg/kg	14.7	18.0	46.6	24.1

In regards to palatability, 6/9 (67%) snakes willingly ate the sausage diet at least once, 4/9 (44%) ate the sausage diet more than once, and only 2/9 (22%) snakes consistently ate the sausage diet (defined as ≥ 5 meals). In comparison, 7/9 (78%) snakes consistently ate the frozen-thawed mouse diet. Willingness to eat the offered diet on a consistent basis was significantly lower for the snakes receiving the sausage diet ($p=0.028$). For snakes that would not willingly eat on a consistent basis, force feeding had to be instituted. Overall, snakes being fed the sausage diet had to be force fed 62% of the time as compared to 24% of the time for snakes being fed the mouse diet.

Unfortunately, regurgitation was a frequent issue post force feeding. One out of every four (25%) meals given by force feeding resulted in partial or complete regurgitation, with 92% of all regurgitation events being associated with force feeding of the sausage diet.

5.4. Discussion

The main objective of this project was to develop a nutritionally-sound diet for snakes and other carnivorous reptiles that could be commercially-produced using alternative non-vertebrate protein sources. The nutritional analysis of the formulated diet is presented in Table 5.1 along with comparisons to other vertebrate meals typically offered to snakes. There were a few differences between the diets, especially in regards to dry matter and protein content, as it was difficult to get protein concentrations up to comparable levels using BSF larvae as the main source of protein. However, the protein content is still within the acceptable ranges for most carnivorous species and the rest of the diet specifications including a lower calorie content were achieved. Snakes in captivity are provided minimal exercise, so obesity is common. While the authors recommend providing exercise for snakes, lowering their calorie intake is another method of reducing the likelihood for obesity.

The benefit of using juvenile snakes for this project was that they eat more frequently, shortening the length of time the experiment needed to run in order to see potential health effects and to better assess the dietary effects on growth. Overall, our hypothesis that there would be no significant differences in health or growth parameters for juvenile snakes being fed these two diets held true. Over the course of two months, all snakes ingesting this insect-based sausage diet showed no ill health effects and were

capable of maintaining and supporting positive growth. Unfortunately, we did have one snake that died over the course of the study, but as was previously mentioned, the cause of death was associated with the act of force feeding the diet and subsequent regurgitation, not with the diet itself.

The only finding that was significant in terms of growth was the change in weight over the course of the experiment. The increased rate of weight gain occurred for both groups of snakes, suggesting that time, and not diet, was responsible for this change. Given the young age of the snakes, the authors believe that this increased rate of weight gain was associated with the snakes being in a logarithmic growth phase, and that both diets were sufficient to meet the caloric needs of the snakes during this time.

The ability of the commercial diet to produce comparable growth rates to a standard mouse diet is evidence that a BSF larvae based diet is capable of providing appropriate nutrition and efficient feed conversion for snakes. Growth data has been investigated in a number of other species being fed either whole BSF larvae or complete feeds containing BSF larvae as the main protein source. In 2007, Bodri and Cole compared the growth rates (body weight and SVL) of hatchling alligators (*Alligator mississippiensis*) fed either whole, dried BSF larvae or a commercialized pellet utilizing fish meal (control) as the main protein source. The alligators were fed the diet for three months. The group fed BSF larvae weighed 41% less than those receiving the pelleted diet. Palatability and decreased feed intake of the dried BSF larvae appeared to be major factors contributing to the results, with the authors suggesting that had the BSF group ingested comparable amounts of food, these alligators would have matched, if not surpassed, the growth of the control group. In 2016, Maurer et al. compared three

complete feeds for laying hens (*Gallus domesticus*). The control diet contained soybean cake as the main protein source, while the two experimental diets used partially defatted BSF meal to replace either 50 or 100% of the soybean cake used in the control diet. After three weeks of feeding, the researchers found no significant differences in body weight, egg weight, or laying performance between any of the diet groups. Similarly, Lock et al. (2016) found that Atlantic salmon (*Salmo salar*) fed diets containing BSF meal as a partial or full replacement for fish meal (25, 50, and 100% fish meal replacement) had similar weight gains as those fed the control diet after 105 days of feeding. However, these findings were only true for one type of BSF meal used. A second BSF meal product made from larvae reared on different substrates and undergoing a different processing method yielded less favorable growth results. The diets containing the second BSF meal led to lower weight gains; this was attributed to lower palatability and feed intake. Several other aquaculture studies have been performed on various fish species, with a majority of the results showing no significant differences in weight gain as long as BSF meal inclusion did not exceed 33%. Palatability and lower feed intake, as well as lower protein digestibility, were cited as being the biggest factors contributing to these results (Barragan-Fonseca et al., 2017).

Digestibility is a measure of the availability of nutrients and is additional evidence to help to determine the nutritive value of food items. Only the soluble portions of the food item are broken down by hydrolysis and other digestive mechanisms, taken up into circulation, and used to supply the body with energy and nutrients necessary for daily functions (Khan et al., 2003). Digestibility can be measured via several methods, but the total collection technique is the most reliable and can be easily performed for small

animals being kept under laboratory conditions. For this technique, the animal is fed known quantities of a food item and all feces are collected for quantification and analysis (Khan et al., 2003). Digestibility of any given nutrient can then be calculated from the apparent digestibility equation provided previously. The resulting digestibility coefficients (Table 5.2) can then be used to compare diet items to each other in order to assess the availability of nutrients and determine the food's nutritive value. To the authors' knowledge, there are no published reports for apparent digestibility in snakes regarding any type of food item. Given the fact that we were only able to collect a small quantity of pooled feces during the course of the experiment, our results should not be considered as a true value for digestibility for either food item, but rather a reference for future studies with larger snakes that provide higher volume samples and as a way to make generalized comparisons to data from other species. Additionally, the digestibility data presented in Table 5.2 takes into account the small alterations that had to be made to the data in order to account for the pinkie skins that were used for scenting on some of the sausages.

Digestibility studies have been performed on a number of different animal species fed diets containing BSF meal as a main ingredient (Bosch et al., 2014; Newton et al., 1977; Renna et al., 2017; Schiavone et al., 2017). Although the information cannot be used as a direct comparison between species or diets due to differences in the chemical and physical composition of the various diets, food processing techniques, and digestive physiologies and feed intake of animals, the previous data can be used to make generalized comparisons between species. The corn snakes in the study were able to digest, on average, 50.85% of the dry matter material from the BSF larvae sausage. This

can be compared to other BSF diets fed to broiler chickens, swine, and rainbow trout, with dry matter digestibilities of 59-63%, 77.5%, and 79%, respectively (Newton, 1977; Renna et al., 2017; Schiavone et al., 2017). The corn snake value appears to be on the low end, but was likely impacted by the digestive physiology of the snakes, as digestion of the higher moisture frozen mouse diet was only 58.4%. For crude protein digestibility, corn snakes were able to digest 77.7% of the protein within the diet, which compares favorably with cats (73-77%), broiler chickens (62%), and rainbow trout (87-91%) (Bosch et al., 2014; Renna et al., 2017; Schiavone et al., 2017). For crude fat or ether extract digestibility, corn snakes digested 94.65% of lipid material as compared to cats at 92-96%, broiler chickens at 93-98%, swine at 83%, and rainbow trout at 97-99% (Bosch et al., 2014; Newton et al., 1977; Renna et al., 2017; Schiavone et al., 2017). Only the swine study reported digestibility data for inorganic feed components, with an ash digestibility of 45%; the corn snakes digested 53.5% of the ash components (Newton et al., 1977). Overall, there does appear to be good agreement between the digestibilities of the various BSF meal complete feed diets and the different species ingesting them.

When comparing the digestibility of our complete feed sausage diet to that of frozen mice, the authors' initial hypothesis was that there would be no significant differences between the two. Unfortunately, we were unable to collect enough feces to prove or disprove significance. However, from the data that was collected, it appears that the frozen mouse and sausage diets were fairly well matched for many of the nutrients studied. The average digestibility of dry matter, crude protein, crude fat, ash, calcium, phosphorous, and potassium were all within a 15% variance, whereas magnesium, zinc, molybdenum, and sulfur had a 21-36% difference in diet digestibility. The remaining

four minerals, including sodium, iron, copper, and manganese, showed much higher differences in nutrient digestibility (180, 80, 188, and 653% difference respectively). Sodium and iron were poorly digested from the sausage diet (sodium=-54.15%; iron=10.4%), while copper and manganese were poorly digested from the frozen mouse diet (copper=20.15%, manganese=-10.1%). Although the differences in digestibility are high, it is not uncommon for mineral digestibility to be more variable than for macronutrients. Mineral absorption is a highly regulated process that can be affected by the mineral's chemical form (organic vs. inorganic), interactions with other dietary components, the host's requirements, and environmental conditions (Wedekind et al., 2010). For example, iron digestibility is affected by the form present in the food, heme iron versus non-heme iron. Heme-iron is generally well absorbed, whereas non-heme iron, which is more common in plant sources, is influenced by other dietary components such as phytates, calcium, phosphorous, manganese, zinc, copper, and ascorbic acid (Wedekind et al., 2010). The lower iron digestibility of the sausage diet may have been related to an increased percentage of non-heme iron due to the presence of more plant ingredients in the BSF diet. Copper absorption can also be affected by excessive calcium or zinc in the diet (Wedekind et al., 2010). The higher levels of zinc for the frozen mouse diet may have resulted in poorer copper absorption within the GI tract of the snakes.

The nutrients with negative digestibility coefficients (sodium and manganese) are the most concerning, as negative values represent more of that nutrient being passed into the feces than was actually ingested. Negative digestibility of sodium has previously been reported after ingestion of BSF larvae. Dierenfeld and King (2008) reported that mountain chicken frogs fed BSF larvae experienced negative digestibility of sodium with

mashed larvae producing higher losses of sodium (digestibility coefficients: -378 ± 64% for whole larvae; -489 ± 78% for mashed larvae). When being fed as whole larvae, other nutrients were also found to produce negative digestibility coefficients (neutral detergent fiber, acid detergent fiber, iron, and molybdenum), but only sodium remained negative and worsened when the larvae were administered as a mashed product. Dierenfeld and King suggested that the BSF larvae may cause some form of gut irritation, potentially leading to a type of diarrhea-like syndrome in the frogs. To the authors' knowledge, no other studies have reported concerns with sodium digestibility when BSF larvae are incorporated as part of a complete feed diet. Collecting blood and measuring sodium concentrations and osmolarity should be considered to determine whether the losses induce hyponatremia or other serum electrolyte imbalances. As for the negative digestibility of manganese from the frozen mouse diet, the authors are unsure as to what significance this holds. Manganese deficiency is rare in humans and animals, and would be extremely unlikely to occur in animals ingesting a whole prey diet. No previous literature could be found for any species regarding the digestibility of manganese or any other trace mineral when consuming a whole prey diet. More data would need to be generated before any conclusions could be drawn from this information.

With the exception of a negative sodium digestibility, we were unable to identify any obvious health risks or growth concerns after two months of feeding the insect-based diet. Therefore, the idea of being able to commercially produce sausage diets with various protein sources could become a reality. However, the largest hurdle to overcome still remains palatability. Prior to the start of this pilot study, a palatability test of the sausage diet was performed using two adult corn snakes and three adult California king

snakes (*Lampropeltis getula californiae*). None of the adult snakes were interested in the plain scented sausage, but after the addition of either mouse blood or powder made from freeze-dried mice, all of the snakes willingly ingested the sausage diet. We initially saw similar results with the juvenile snakes, with the exception of two snakes that refused all meals during the entire course of study. Sixty-seven percent (6/9) of the snakes willingly ingested the sausage diet at least once. However, as time went on, only two of the snakes continued to eat the sausage diet. Unlike the adult snakes that ingested the diet with either blood or freeze-dried mouse powder scent added, all of the juvenile snakes, except one, required the sausage to be covered in the skin of a pinkie mouse before they would consider eating the sausage. Given that juvenile snakes have a tendency to be selective eaters, the authors believe that a repeat of this study using only adult snakes would provide more insight concerning the palatability of this novel diet.

There were a number of limitations to this study that should be addressed. Initially, the authors planned on using adult snakes for this study, but our inability to acquire adult corn snakes in a timely manner, led to us using juvenile snakes instead. The juvenile snakes did provide us with the opportunity to gather data regarding growth, but their small size prevented us from being able to safely collect blood to perform hematological and biochemical analyses to assess whether differences in digestibility had an impact on the physiology of the snakes. The small size of the snakes also limited the amount of feces available for analysis. Larger sample quantities would have allowed for replicates to be collected, refining the authors ability to compare the two diets. Another limitation associated with using juvenile snakes was that as the snakes grew over the course of the study, the type of prey offered changed. To maintain the standard of

offering the snakes a diet consistent with 15% of their body weight, the prey changed from pinkie to fuzzy-sized mice. Table 5.1 illustrates the nutritional profiles for both life stages (neonatal mice vs. juvenile mice). This change in diet did not allow for a perfect cross-over design when comparing diets in regard to impacts on growth or digestibility, and is why we used a mixed model to assess the effect of time and diet. The addition of pinkie skins to the sausages to entice a feeding response also altered the nutritional profile of the ingested diet. However, the digestibility calculations were adjusted to account for the nutritional content of the skins. Finally, the regurgitation noted during the study was also a limitation. To be able to compare the diets' effect on growth on a head to head basis, the snakes needed to ingest the same amount of food per unit of body weight. With the snakes not eating on their own, force feeding had to be instituted.

Unfortunately, approximately one out of every four meals given by force feeding resulted in either partial or complete regurgitation. Due to the thicker nature of the sausage material compared to the pureed mice, the sausage diet was much easier for the snakes to regurgitate, leading to unequal ingestion of the diets (percent body weight ingested: sausage diet=10.6%, mouse diet=13.2%). Again, to account for this, we used a repeated measures mixed linear model to analyze the results. The lack of significance in the model by diet type suggests that regurgitation did not negatively impact the results. It is important to note that while these limitations were encountered, it remained important to have performed a study assessing how juvenile snakes would respond to this novel diet.

5.4.1. Conclusions

The result of this pilot study suggest that there does not appear to be any short term health problems or growth deficiencies associated with a BSF larvae-based sausage diet in juvenile corn snakes; however, further research is needed to address issues with palatability and long term health. If the authors can adequately address the issues with palatability, sausage diets made from novel protein sources could be a viable option for increasing diet variety for captive snakes and other carnivorous reptiles.

CHAPTER 6.
CONCLUSIONS

The results of this work suggest that BSF larvae do have a positive nutritive value and an inherent ability to support health and growth of reptiles. However, in their natural state, they are not capable of providing sufficient calcium, sodium, or fat soluble vitamins. Gut loading of the larvae with vitamin A proved to be a successful and consistent process, as long as certain environmental conditions were held constant. It stands to reason that a more complete gut loading diet with additional nutrients could be utilized to further supplement the larvae and ultimately the reptile.

However, to be able to utilize gut loaded nutrients, the reptile species in question must first be able to digest the BSF larvae. As was seen with leopard geckos, species of reptiles that are more apt to chew their prey should be better at digesting the larvae compared to what was previously published for mountain chicken frogs. Digestibility of a gut loaded nutrient was proven with vitamin A, given the increased plasma and liver vitamin A concentrations in the geckos receiving the gut loaded larvae. This suggests that other nutrients could be supplemented through gut loading as well. This is fortunate, as there are several other nutrients (vitamins D_3 and E, calcium, sodium, and protein) that need to be supplemented in order to provide a healthy nutritional profile. In the case of calcium, it remained bound within the exoskeleton matrix and undigested, despite the increased disruption to the exoskeleton through mastication. Without calcium supplementation through dusting or gut loading, stores within the body may decline over time, as was noted in the leopard geckos' plasma calcium concentrations. Similarly, total protein and sodium may also decline, but further research is needed to confirm if any of the changes are clinically significant.

When fed as a complete meal, these nutrients of concern become less of an issue as they can be directly supplemented to the formulated diet. Overall, long-term health becomes the main focus. For the corn snakes receiving the sausage diet, no changes in health were seen and growth was adequately maintained. The ability of the sausage diet to produce comparable growth rates to a standard mouse is evidence that a BSF larval based diet is capable of providing appropriate nutrition and efficient feed conversion for snakes. The primary issue to overcome with the BSF sausage is palatability.

Altogether, these projects positively support the use of BSF larvae as a component of captive reptile diets. There is no such thing as the "perfect insect," and no food item should ever be fed exclusively. Varying a diet is the best way to support captive reptiles and help meet their full nutritional needs, but supplementation through gut loading or with other ingredients in a complete feed should be able to improve the nutritional value of the BSF larvae.

REFERENCES

Abate AL, Coke R, Ferguson G, Reavill D. 2003. Chameleons and vitamin A. J Herp Med Surg, 13(2):23-31.

Alberton S, Cojean O, Froment R, Maccolini E, Vergneau-Grosset C. 2018. Determination of leopard gecko (*Eublepharis macularius*) packed cell volume and plasma biochemistry reference intervals and reference values. Proc ARAV, 933-934.

Allen, M.E. 1997. From blackbirds and thrushes… to the gut-loaded cricket: a new approach to zoo animal nutrition. *Brit J Nutr*, 78(S2):S135-S143.

Ariel E, Ladds PW, Buenviaje GN. 1997. Concurrent gout and suspected hypovitaminosis A in crocodile hatchlings. Aust Vet J, 75(4):247-249.

Attard, L. 2013. The development and evaluation of a gut loading diet for feeder crickets formulated to provide a balanced nutrient source for insectivorous amphibians and reptiles (Master's thesis). University of Guelph, Guelph, Ontario. p.149

Barragan-Fonseca KB, Dicke M, van Loon JJA. 2017. Nutritional value of the black soldier fly (*Hermetia illucens L*.) and its suitability as animal feed- a review. J Insects Food Feed, 3(2):105-120.

Berkvens CN, Lentini A, Dutton CJ, Pearl DL, Barker IK, Crawshaw GJ. 2014. Serum and hepatic vitamin A levels in captive and wild marine toads (*Bufo marinus*). Zoo Biol 33(6):536-543.

Bodri MS, Cole ER. 2007. Black soldier fly (*Hermetia illucens* Linnaeus) as feed for American alligator (*Alligator mississippiensis daudin*). GA J Sci, 65(2):82-88.

Bondari K, Sheppard DC. 1981. Soldier fly larvae as feed in commercial fish production. Aquaculture, 24:103-109.

Bosch G, Zhang S, Oonincx DGAB, Hendriks WH. 2014. Protein quality of insects as potential ingredients for dog and cat foods. J Nutr Sci, 3(29):1-4.

Boyer TH. 2019. Hypovitaminosis and hypervitaminosis A. *In* Divers SJ and Stahl SJ (eds): Mader's Reptile and Amphibian Medicine and Surgery. Elsevier, St. Louis, MO:1316-1317.

Boyer TH, Scott PW. 2019. Nutrition. *In* Divers SJ and Stahl SJ (eds.): Mader's Reptile and Amphibian Medicine and Surgery. Elsevier, St. Louis, MO:201-223.

Boyer TH, Scott PW. 2019. Nutritional Secondary Hyperparathyroidism. In Divers SJ and Stahl SJ (eds.): Mader's Reptile and Amphibian Medicine and Surgery. Elsevier, St. Louis, MO:1326-1327.

Boykin KL, Butler-Perez K, Mitchell MA. 2018. Evaluation of vitamin A gut loading in black soldier fly larvae. Proc ARAV, 981.

Boykin KL, Butler-Perez K, Mitchell MA. 2018. Feeding trial and health assessment of corn snakes (*Pantherophis guttatus*) fed an insect-based sausage diet. Proc ARAV, 975.

Caligiani A, Marseglia A, Leni G, Baldassarre S, Maistrello L, Dossena A, Sforza S. 2018. Composition of black soldier fly prepupae and systematic approaches for extraction and fractionation of protein, lipids and chitin. Food Res Int, 105:812-820.

Calle PP, Rivas J, Munoz M, Thorbjarnarson J, Dierenfeld ES, Holmstrom W, Braselton WE, Karesh WB. 1994. Health assessment of free-ranging anaconda (*Eunectes murinus*) in Venezuela. J Zoo Wildl Med, 25(1):53-62.

Cammack JA, Tomberlin JK. 2017. The impact of diet protein and carbohydrate on select life-history traits of the black soldier fly *Hermetia illucens* (L.) (Diptera:Stratiomyidae). Insects, 8(2):56.

Chaffin K, Norton TM, Gilardi K, Poppenga R, Jensen JB, Moler P, Cray C, Dierenfeld ES, Chen T, Oliva M, Origgi FC, Gibbs S, Mazzaro L, Mazet J. 2008. Health assessment of free-ranging alligator snapping turtles (*Macrochelys temminckii*) in Georgia and Florida. J Wildl Dis, 44(3):670-686.

Dabbou S, Gai F, Biasato I, Capucchio MT, Biasibetti E, Dezzutto D,…Schiavone A. 2018. Black soldier fly defatted meal as a dietary protein source for broiler chickens: Effects on growth performance, blood traits, gut morphology and histological features. J Anim Sci Biotech, 9(1):1–10.

D'Ambrosio DN, Clugston RD, Blaner WS. 2011. Vitamin A metabolism: An update. Nutrients, 3(1):63-103.

Deem SL, Dierenfeld ES, Sounguet GP, Alleman AR, Cray C, Poppenga RH, Norton TM, Karesh WB. 2006. Blood values in free-ranging nesting leatherback sea turtles (*Dermochelys coriacea*) on the coast of the Republic of Gabon. J Zoo Wildl Med, 37(4):464-471.

Delany MF. 1990. Late summer diet of juvenile American alligators. J Herpetol, 24(4):418-421.

De Marco M, Martínez S, Hernandez F, Madrid J, Gai F, Rotolo L,…Schiavone A. 2015. Nutritional value of two insect meals (*Tenebrio molitor* and *Hermetia illucens*) for broiler chickens: apparent nutrient digestibility, apparent ileal amino acid digestibility and apparent metabolizable energy. Anim Feed Sci Technol, 209:211–218.

De Voe RS. 2014. Nutritional support of reptile patients. Vet Clin Exot Anim, 17(2):249-261.

Diener S, Zurbrugg C, Tockner K. 2009. Conversion of organic material by black soldier fly larvae: establishing optimal feeding rates. Waste Manag Res, 27(6):603-610.

Dierenfeld ES, Alcorn HL, Jacobsen KL. 2002. Nutrient composition of whole vertebrate prey (excluding fish) fed in zoos. Nat Agric Libr, Z7994, Z65.

Dierenfeld ES, King J. 2008. Digestibility and mineral availability of phoenix worms, *Hermetia illucens*, when fed to mountain chicken frogs, *Leptodactylus fallux*. J Herp Med Surg, 18(3/4):100-105.

Donoghue S. 2006. Nutrition. *In* Mader DR (ed): Reptile Medicine and Surgery. Elsevier, St. Louis, MO:251-298.

Dutton C, Lentini A, Berkvens C, Crawshaw G. 2014. The effect of supplementation with vitamin A on serum and liver concentrations in Puerto Rican crested toads (*Peltophryne lemur*) and its lack of impact on brown skin disease. Zoo Biol 33(6):553-557.

Erickson MC, Islam M, Sheppard C, Liao J, Doyle MP. 2004. Reduction of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Enteritidis in chicken manure by larvae of the black soldier fly. J Food Prod, 67(4): 685-690.

Fatchurochim S, Geden CJ, Axtell RC. 1989. Filth fly (Diptera) oviposition and larval development in poultry manure of various moisture levels. J Entomol Sci, 24(2):224-231.

Finke MD. 2002. Complete nutrient composition of commercially raised invertebrates used as food for insectivores. Zoo Biol, 21(3):269-285.

Finke MD. 2003. Gut loading to enhance the nutrient content of insects as food for reptiles: a mathematical approach. Zoo Biol, 22(2):147-162.

Finke MD. 2007. Estimate of chitin in raw whole insects. Zoo Biol, 26(2):105-115.

Finke MD. 2013. Complete nutrient content of four species of feeder insects. Zoo Biol, 32(1):27-36.

Finke MD, Dunham SU, Kwabi CA. 2005. Evaluation of four dry commercial gut loading products for improving the calcium content of crickets, *Acheta domesticus*. J Herp Med Surg, 15(1):7-12.

Funk RS. 2006. Snakes. *In* Mader DR (ed): Reptile Medicine and Surgery. Elsevier, St. Louis, MO:42-58.

Guimarães IG, Pezzato LE, Barros MM, Fernandes RDN. 2012. Apparent nutrient digestibility and mineral availability of protein-rich ingredients in extruded diets for Nile tilapia. Revista Brasileira de Zootecnia, 41(8):1801–1808.

Gullan PJ, Cranston, PS. 2014. Insect development and life histories. In Gullan PJ and Cranston PS (Eds.), The Insects: An Outline of Entomology. Wiley Blackwell, Inc., West Sussex, UK:253-301.

Hale OM. 1973. Dried *Hermetia illucens* larvae (Diptera: Stratiomyidae) as a feed additive for poultry. J GA Entomol Soc, 8:16-20.

Harker KS, Lane C, De Pinna E, Adak GK. 2011. An outbreak of *Salmonella typhimurium* DT191a associated with reptile feeder mice. Epidemiol Infect, 139(8): 1254-1261.

Holmes L, VanLaerhoven S, Tomberlin J. 2016. Lower temperature threshold of black soldier fly (Diptera: Stratiomyidae) development. J Insects Food Feed, 2(4):255-262.

Holladay SD, Wolf JC, Smith SA, Jones DE, Robertson JL. 2001. Aural abscesses in wild-caught box turtles (*Terrapene carolina*): Possible role of organochlorine-induced hypovitaminosis A. Ecotoxicol Environ Saf, 48(1):99-106.

Hume EM, Krebs HA. 1949. Vitamin A requirement of human adults: An experimental study of vitamin A deprivation in man. Med Res Counc Spec Rep Series, 264:138-140

Hunt Coslik A, Ward AM, McClements RD. 2009. Gut loading as a method to effectively supplement crickets with calcium and vitamin A. Proc Zoo Wildl Nutr, AZA NAG, Tulsa, OK.

Inês J, Fernandes M, Murakami AE. 2010. Arginine metabolism in uricotelic species. Anim Sci Maringá, 32(4):357–366.

Johannsen OA. 1922. Stratiomyiid larvae and puparia of the north eastern states. J NY Entomol Soc, 30(4):141-153.

Khan, MA, Mahr-Un-Nisa, Sarwar, M. 2003. Techniques measuring digestibility for the nutritional evaluation of feeds. Int J Agri Biol, 5(1):91-94.

Klaphake E. 2010. A fresh look at metabolic bone disease in reptiles and amphibians. Vet Clin Exot Anim, 13(3):375-392.

Knafo SE, Norton TM, Mitchell MA, Stevenson DJ, Hyslop N, Poppenga R,…Dierenfeld ES. 2016. Health and nutritional assessment of free-ranging eastern indigo snakes (*Drymarchon couperi*) in Georgia, United States. J Zoo Wild Med, 47(4):1000-1012.

Lalander C, Diener S, Magri ME, Zurbrugg C, Lindstrom A, Vinneras B. 2013. Faecal

sludge management with the larvae of the black soldier fly (Hermetia illucens)- From a hygiene aspect. Sci Total Environ, 458-460C:312-318.

Lalander CH, Fidjeland J, Diener S, Eriksson S, Vinneras B. 2015. High waste-to-biomass conversion and efficient *Salmonella* spp. reduction using black soldier fly for waste recycling. Agron Sustain Dev, 35(1): 261-271.

Latney L, Clayton LA. 2014. Updates on amphibian nutrition and nutritive value of common feeder insects. Vet Clin Exotic Anim, 17(3): 347-367.

Lee KM, McReynolds JL, Fuller CC, Jones B, Herman TJ, Byrd JA, Runyon M. 2008. Investigation and characterization of the frozen feeder rodent industry in Texas following a multi-state *Salmonella typhimurium* outbreak associated with frozen vacuum-packed rodents. Zoonoses Public Health, 55(8-10):488-496.

Li H, Vaughan MJ, Browne RK. 2009. A complex enrichment diet improves growth and health in the endangered Wyoming toad (*Bufo baxteri*). Zoo Biol, 28(3):197-213.

Livingston S, Lavin SR, Sullivan K, Attard L, Valdes EV. 2014. Challenges with effective nutrient supplementation for amphibians: A review of cricket studies. Zoo Biol, 33(6):483-592.

Lock ER, Arsiwalla T, Waagbo R. 2016. Insect larvae meal as an alternative source of nutrients in the diet of Atlantic salmon (*Salmo salar*) postmolt. Aquacult Nutr, 22(6):1202-1213.

Makkar HPS, Tran G, Heuzé V, Ankers P. 2014. State-of-the-art on use of insects as animal feed. Anim Feed Sci Technol, 197(1):1–33.

Mans C, Braun J. 2014. Update on common nutritional disorders of captive reptiles. Vet Clin Exot Anim, 17(3):369-395.

Marono S, Loponte R, Lombardi P, Vassalotti G, Pero ME, Russo F, …Bovera F. 2017. Productive performance and blood profiles of laying hens fed *Hermetia illucens* larvae meal as total replacement of soybean meal from 24 to 45 weeks of age. Poultry Sci, 96(6):1783–1790.

Maurer V, Holinger M, Amsler Z, Früh B, Wohlfahrt J, Stamer A, Leiber F. 2016. Replacement of soybean cake by *Hermetia illucens* meal in diets for layers. J Insects Food Feed, 2(2):83-90.

McCarthy PT, Cereoedo LR. 1952. Vitamin A deficiency in the mouse. J Nutr, 46(3):361-376.

Mitchell MA. 2009. Snakes. *In* Mitchell MA, Tully TN (eds): Manual of Exotic Pet Practice. Elsevier, St. Louis, MO:136-163.

National Resource Council (NRC). (1994). Nutrient requirements of poultry. Washington, DC: National Academy Press.

National Resource Council (NRC). (1995). Nutrient requirements of laboratory animals. Washington, DC: National Academy Press.

Newton GL, Booram CV, Barker RW, Hale OM. 1977. Dried *Hermetia illucens* larvae meal as a supplement for swine. J Anim Sci, 44(3):395-400.

Oonincx DGAB, Dierenfeld ES. 2012. An investigation into the chemical composition of alternative invertebrate prey. Zoo Biol, 31(1):40-54.

Oonincx DGAB, van Broekhoven S, van Huis A, van Loon JJA. 2015. Feed conversion, survival and development, and composition of four insect species on diets composed of food by-products. PLoS ONE, 10(12): e0144601.

Oyarzun SE, Crawshaw GJ, Valdes EV. 1996. Nutrition of the tamandua: I. Nutrient composition of termites (*Nasutitermes* spp.) and stomach contents from wild tamanduas (*Tamandua tetradactyla*). Zoo Biol, 15(5):509-524.

Pennino M, Dierenfeld ES, Behler JL. 1991. Retinol, α-tocopheral and proximate nutrient composition of invertebrates used as feed. Int Zoo Yearb. 30(1):143-149.

Phoenix Worms® The Superior Calcium-Rich Feeder. (2019, February 23). Retrieved from https://phoenixworm.com/

Rachmawati, Buchori D, Hidayat P, Hem S, Fahmi DMR. 2010. Development and nutritional content of *Hermetia illucens* (Linnaeus) (Diptera: Stratiomyidae) larvae on oilpalm kernel. J Entomol Indon, 7(1):28-41.

Raila J, Schumacher A, Gropp J, Schweigert FJ. 2002. Selective absorption of carotenoids in the common green iguana (*Iguana iguana*). Comp Biochem Physiol A, 132(2):513-518.

Raiti P. 2019. Endocrinology. In Divers SJ and Stahl SJ (eds.): Mader's Reptile and Amphibian Medicine and Surgery. Elsevier, St. Louis, MO:835-848.

Raphael BL, Klemens MW, Moehlman P, Dierenfeld ES, Karesh WB. 1994. Blood values in free-ranging pancake tortoises (*Malacochersus tornieri*). J Zoo Wildl Med, 25(1):63-67.

Renna M, Schiavone A, Gai F, Dabbou S, Lussiana C, Malfatto V,…Gasco L. 2017. Evaluation of the suitability of a partially defatted black soldier fly (*Hermetia illucens* L.) larvae meal as ingredient for rainbow trout (*Oncorhynchus mykiss* Walbaum) diets. J Anim Sci Biotech, 8:57.

Roll, U., Feldman, A., Novosolov, M., Allison, A., Bauer, A. M., Bernard, R.,…Meiri, S. 2017. The global distribution of tetrapods reveals a need for targeted reptile conservation. Nature Ecology and Evolution, 1(11):1677–1682.

Schiavone A, De Marco M, Martínez S, Dabbou S, Renna M, Madrid J,…Gasco L. 2017. Nutritional value of a partially defatted black soldier fly larvae (*Hermetia illucens* L.) meal for broiler chickens: apparent nutrient digestibility, apparent metabolizable energy and apparent ileal amino acid digestibility. J Anim Sci Biotech, 8:51.

Schweigert FJ, Uehlein-Harrell S, Hegel GV, Wiesner H. 1991. Vitamin A (retinol and retinyl esters), α-tocopherol and lipid levels in plasma of captive wild mammals and birds. J Vet Med A, 38(1-10):35-42.

Sheppard CD, Tomberlin JK, Joyce JA, Kiser BC, Sumner SM. 2002. Rearing methods for the black soldier fly (Diptera: Stratiomyidae). Journal of Medical Entomology, 39(4):695-698.

Spranghers T, Ottoboni M, Klootwijk C, Ovyn A, Debroosere S, De Meulenaer B,…De Smet S. 2017. Nutritional composition of black soldier fly (*Hermetia illucens*) prepupae reared on different organic waste substrates. J Sci Food Agric, 97(5):2594-2600.

Sullivan KE, Fleming G, Terell S, Smith D, Ridgley F, Valdes EV. 2014. Vitamin A values of wild-caught Cuban tree frogs (*Osteopilus septentrionalis*) and marine toads (*Rhinella marina*) in whole body, liver, and serum. J Zoo Wild Med, 45(4):892-896.

Stahl SJ, DeNardo DF. 2019. Theriogenology. In Divers SJ and Stahl SJ (eds.): Mader's Reptile and Amphibian Medicine and Surgery. Elsevier, St. Louis, MO:849-893.

Tomberlin JK, Adler PH, Myers HM. 2009. Development of the black soldier fly (Diptera: Stratiomyidae) in relation to temperature. Environ Entomol, 38(3):930-934.

Tomberlin JK, Sheppard DC, Joyce JA. 2002. Selected life-history traits of black soldier flies (Diptera:Stratiomyidae) reared on three artificial diets. Ann Entomol Soc Am, 95(3):379-386.

van der Loo B, Labugger R, Aebischer CP, Bachschmid M, Spitzer V, Kilo J,…Luscher TF. 2004. Age-related changes in vitamin A status. J Cardiovasc Pharmacol Ther, 43(1):26-30.

Wallace PA, Nyameasem JK, Adu-Aboagye GA, Affedzie-Obresi S, Nkegbe EK, Karbo N, …Maquart PO. (2017). Impact of black soldier fly larval meal on growth performance, apparent digestibility, haematological and blood chemistry indices of guinea fowl starter keets under tropical conditions. Trop Anim Health Prod, 49(6): 1163–1169.

Wedekind KJ, Kats L, Yu S, Paetau-Robinson I, Cowell CS. 2010. Micronutrients:

minerals and vitamins. *In* Hand MS, Thatcher CD, Remillard RL, Roudebush P, Novtony BJ (eds): Small Animal Clinical Nutrition. Mark Morris Institute, Topeka, KS:107-148.

Wiggans KT, Sanchez-Migallon Guzman D, Reily CM, Vergneau-Grosset C, Kass PH, Hollingsworth SR. 2018. Diagnosis, treatment, and outcome of and risk factors for ophthalmic disease in leopard geckos (*Eublepharis macularius*) at a veterinary teaching hospital: 52 cases (1985-2013). JAVMA, 252(3):316-323.

Woodring JP. 1983. Control of moulting in the house cricket, *Acheta domesticus*. J Insect Physiol, 29(6):461-464.

VITA

Kimberly Lynne Boykin was born and raised in North Carolina by her parents Linda Legg and Craig Boykin. She graduated Summa Cum Laude from Florida State University with a Bachelor of Science degree in Biological Sciences in 2008. She completed the requirements for the degree of Doctor of Veterinary Medicine in 2016 from the College of Veterinary Medicine at North Carolina State University, Raleigh, NC. She was then chosen as an intern in the small animal medicine and surgery program at the School of Veterinary Medicine at Louisiana State University for 2016-2017. In 2017, she was accepted into the graduate program in the Department of Veterinary Clinical Sciences, School of Veterinary Medicine, Louisiana State University. She is expected to graduate with a Master of Science degree in May 2019 and to continue on at Louisiana State University in pursuit of a PhD degree.